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THE EXPRESSION AND CELLULAR LOCALIZATION OF CC-CHEMOKINE RECEPTOR 5 (CCR5) AFTER TRAUMATIC BRAIN INJURY

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**THE EXPRESSION AND CELLULAR LOCALIZATION OF
CC-CHEMOKINE RECEPTOR 5 (CCR5)
AFTER TRAUMATIC BRAIN INJURY**

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
And
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

By

VUVI HOANG NGUYEN, B.A.
Houston, Texas

MAY, 2010

**THE EXPRESSION AND CELLULAR LOCALIZATION OF
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This is dedicated to my loving family:
my dad Khoa Nguyen, my mom Dung Hoang,
and my little sister Thy-Thy Nguyen.

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constant reminder that I am her role model. This position pushes me even further to achieve all my hopes, dreams, and happiness in my own life.

The Expression and Cellular Localization of CC-Chemokine Receptor

5 (CCR5) after Traumatic Brain Injury

Vuvi H. Nguyen

Supervisory Professor: Dr. Andrew Bean, Ph.D.

Traumatic brain injury results from a primary insult and secondary events that together result in tissue injury. This primary injury occurs at the moment of impact and damage can include scalp laceration, skull fracture, cerebral contusions and lacerations as well as intracranial hemorrhage. Following the initial insult, a delayed response occurs and is characterized by hypoxia, ischemia, cerebral edema, and infection. During secondary brain injury, a series of neuroinflammatory events are triggered that can produce additional damage but may also help to protect nervous tissue from invading pathogens and help to repair the damaged tissue. Brain microglia and astrocytes become activated and migrate to the site of injury where these cells secrete immune mediators such as cytokines and chemokines.

CC-chemokine receptor 5 (CCR5) is a member of the CC chemokine receptor family of seven transmembrane G protein coupled receptors. CCR5 is expressed in the immune system and is found in monocytes, leukocytes, memory T cells, and immature dendritic cells. Upon binding to its ligands, CCR5 functions in the chemotaxis of these immune cells to the site of inflammation. In the CNS, CCR5 and its ligands are expressed in multiple cell types. In this study, I investigated whether CCR5 expression is altered in brain after traumatic brain injury. I examined the time course of CCR5 protein expression in cortex and hippocampus using quantitative western analysis of tissues from injured rat brain after mild impact injury. In addition, I also investigated the cellular localization of CCR5 before and after brain injury using confocal microscopy. I have observed that after brain injury CCR5 is upregulated in a time dependent manner in neurons of the parietal cortex and hippocampus. The absence of CCR5

expression in microglia and its delayed expression in neurons after injury suggests a role for CCR5 in neuronal survival after injury.

TABLE OF CONTENTS

DEDICATIONS	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	vi
INTRO I: BRAIN INJURY TRIGGERS NEUROINFLAMMATION	1
1.1 BLOOD BRAIN BARRIER DISRUPTION	1
1.2 ROLE OF CYTOKINES DURING NEUROINFLAMMATION	6
INTRO II: CHEMOKINES AND CHEMOKINE RECEPTORS	7
2.1 THE STRUCTURE AND FUNCTION OF CHEMOKINES	7
2.2. STRUCTURE OF CHEMOKINE RECEPTORS	8
2.3. CHEMOKINE RECEPTOR SIGNALING	12
INTRO III: INTRODUCTION OF CCR5	14
3.1 CC-CHEMOKINE RECEPTOR 5 (CCR5)	14
3.2 CCR5 SERVES AS CO-RECEPTOR FOR HIV	14
3.3. TRAFFICKING OF CCR5	15
3.4 CCR5 SIGNALING	17
INTRO IV: CHEMOKINES AND CHEMOKINE RECEPTORS IN THE CNS	21
4.1 CCR5 IN BRAIN DEVELOPMENT	21
4.2 CCR5 EXPRESSION IN THE CNS	23
MATERIALS AND METHODS	24
5.1. ANIMALS	24
5.2 ANTIBODIES	24
5.3. CONTROLLED CORTICAL IMPACT INJURY	24
5.4 SAMPLE PREPARATION	25

5.5 WESTERN ANALYSIS	25
5.6. IMMUNOHISTOCHEMISTRY	25
5.7. STATISTICAL ANALYSIS	26
5.8. PLASMIDS, CELL CULTURE TREATMENT, & TRANSFECTIONS	26
5.9. IMMUNOFLUORESCENCE MICROSCOPY	27
RESULTS	28
6.1 TIME COURSE OF CCR5 PROTEIN EXPRESSION IN CORTEX AND HIPPOCAMPUS	28
6.2 CCR5 IS LOCALIZED IN NEURONS IN CORTEX AFTER TBI	32
6.3 CCR5 IS LOCALIZED IN NEURONS IN HIPPOCAMPUS AFTER TBI	41
6.4 NIH ANTIBODY CONTROL EXPERIMENT	51
DISCUSSION	53
CONCLUSION	56
BIBLIOGRAPHY	57
VITA	67

LIST OF FIGURES

Figure 1	Time line of Traumatic Brain Injury	2
Figure 2	Blood-Brain Barrier dysfunction following TBI	5
Figure 3	CC-Chemokine Receptor Family	10
Figure 4	Schematic Representation of the CCR5 sequence	11
Figure 5	Signaling pathways downstream of chemokine receptor activation	13
Figure 6	JAK/STAT Signaling Pathway	19
Figure 7	Time course of CCR5 protein expression in cortex after TBI	30
Figure 8	Time course of CCR5 protein expression in hippocampus after TBI	31
Figure 9	Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI: CCR5 and MAP2	33
Figure 10	Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI: CCR5 and GFAP	36
Figure 11	Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI: CCR5 and IBA-1	39
Figure 12	CCR5 is localized in neurons in hippocampus after TBI: Sham	43
Figure 13	CCR5 is localized in neurons in hippocampus after TBI: 3 hours	45
Figure 14	CCR5 is localized in neurons in hippocampus after TBI: 3 days	47
Figure 15	CCR5 is localized in neurons in hippocampus after TBI: 28 days	49
Figure 16	NIH CCR5 antibody control experiment	52

INTRODUCTION I:

Brain Injury Triggers Neuroinflammation

Traumatic brain injury results in tissue damage that is initially caused by mechanical disruption. This primary injury occurs at the moment of impact and damages can include scalp laceration, skull fracture, cerebral contusions and lacerations as well as intracranial hemorrhage. A delayed response, known as secondary brain injury, occurs following the initial insult and is characterized by hypoxia, ischemia, cerebral edema and infection. Secondary brain injury involves the activation of a series of neuroinflammatory events that can produce additional damage. However, these secondary events may also serve to protect nervous tissue from invading pathogens and help to repair damaged tissue (1).

The inflammatory response that occurs during secondary brain injury is characterized by a cascade of events that induce the activation of glial cells, recruitment of leukocytes, upregulation of endothelial adhesion molecules, and upregulation of immune mediators such as cytokines and chemokines (2). The time course of the physiopathological events that occur after brain injury vary and are initiated from minutes to hours after injury and span from days to weeks, depending upon the the intensity of brain damage (Figure 1).

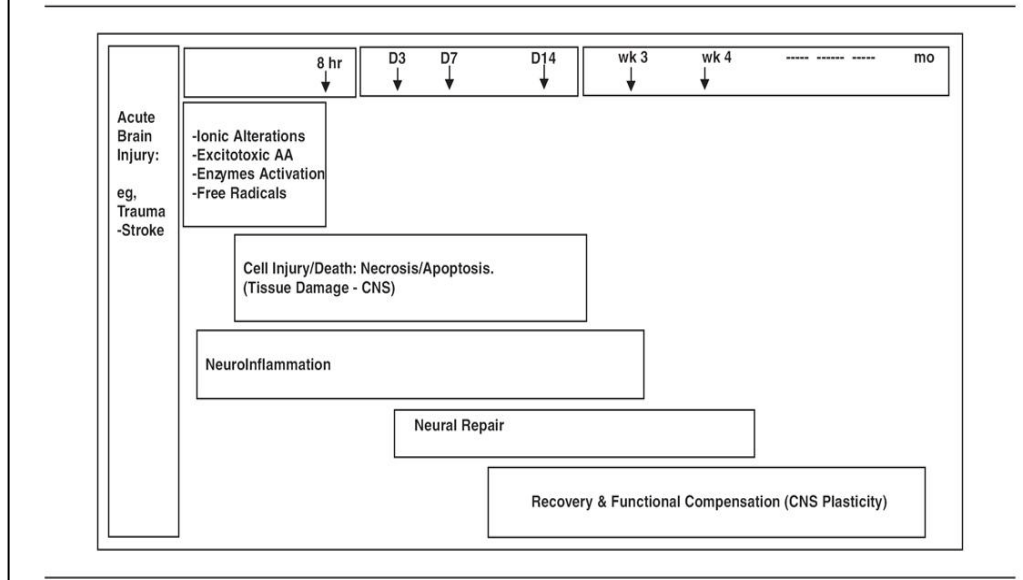


Fig 1: “Schematic drawing depicting dynamics involved in the main physiopathological events triggered by acute brain injury, their relative sequences, and their estimated time course. It also illustrates how better knowledge of timing might have implications for more effective interventions (therapeutic windows of opportunity). CNS indicates central nervous system. AA indicates amino acid.” (Kadhim et al. 2008)

“Reprinted from *Journal of Intensive Care Medicine*, Vol 4, Hazim J. Kadhim, Jean Duchateau, and Guillaume Sebire, *Cytokines and Brain Injury*, 236-249, 2008, with permission from Elsevier.”

1.1 BLOOD-BRAIN BARRIER DISRUPTION

Injury-induced disruption of the blood brain barrier (BBB) contributes to neuroinflammation by promoting migration of immune cells across the BBB and travel towards the injured site (3). The BBB helps to exclude circulating blood cells found in the vascular system from entering the parenchyma of the central nervous system (CNS). Following brain injury, the blood-brain barrier is disrupted allowing circulating blood cells such as neutrophils, monocytes, and lymphocytes to enter the CNS and travel towards the injured site. Inside the brain parenchyma, leukocytes secrete pro-inflammatory cytokines, oxygen radicals, and nitric oxide that can promote cell damage. Simultaneously, glial cells can release trophic factors to promote cell survival. The presence of damaged cells and debris that are found in the brain parenchyma results in activation of resting astrocytes and microglia. These activated cells undergo changes in their morphology and function. For example, activated microglia can promote tissue remodeling as well as attenuate inflammation by acting as phagocytes to engulf dead cells and tissue debris. On the other hand, activated microglia cells are also capable of increasing tissue damage by releasing various neurotoxic substances such as reactive oxygen and nitrogen species. This release of free radicals can activate apoptotic pathways that may result in cell injury/death (4). Activated astrocytes may also have dual actions during inflammation. Activated astrocytes produce excess glial fibrillary acidic protein (GFAP) and form glial scars that replace damaged tissues and isolate damaged areas. This can “restrict neural repair and act as a barrier against axonal regeneration” (5). Reactive astrocytes can also play neuroprotective roles by producing neurotrophic factors such as neural growth factor (NGF) and brain-derived growth factor (BDGF) that can help stimulate axonal growth and promote tissue repair. Thus, resident glial cells and neurons can synthesize cytokines, chemokines, and other trophic factors that can exert damaging or protective effects on

neighboring cells. Figure 2 (Ziebell and Morganti-Kossmann 2010) illustrates the inflammatory events that occur following the disruption of the blood-brain barrier after injury.

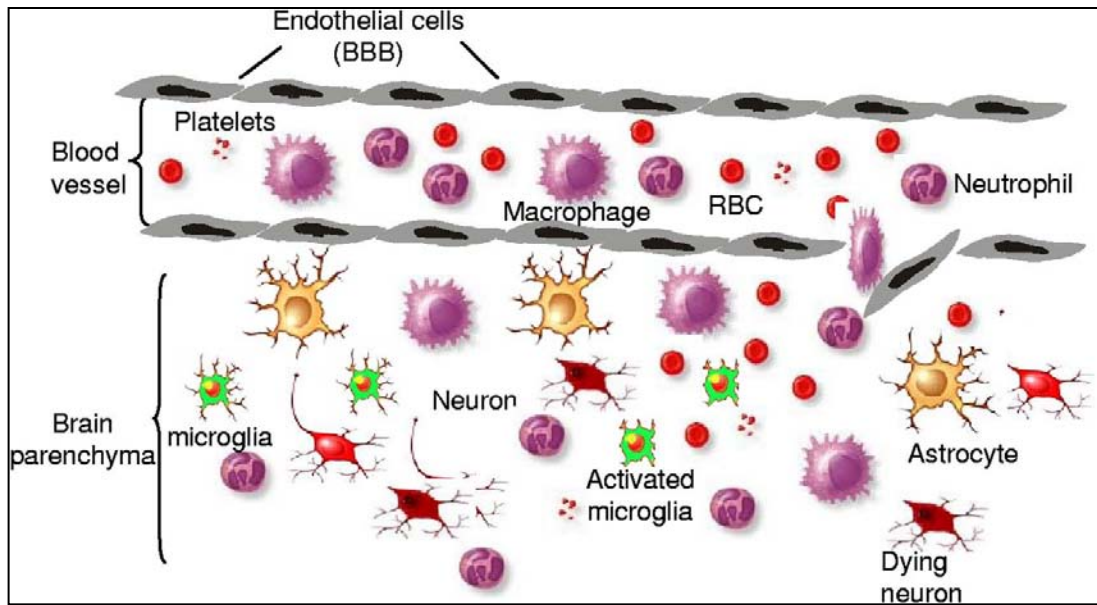


Fig 2: “Blood–brain barrier (BBB) dysfunction following traumatic brain injury. The BBB is the physical interface between the brain parenchyma and the vascular system. It consists of tightly placed endothelial cells, which regulate the passage of cells, molecules, and ions into the brain tissue. Following brain injury, the integrity of the BBB is disrupted, resulting in the infiltration of red blood cells (RBCs) and white blood cells into the parenchyma, thus allowing communication between the peripheral and resident immune cells; this communication is mediated by the release and exchange of cytokines and other toxic or neurotrophic factors. Some of these mediators can lead to the activation of glial cells, as well as neuronal cell death, whereas others promote regenerative mechanisms of the damaged brain.” (Ziebell and Morganti-Kossmann 2010) (14)

“Reprinted from Neurotherapeutics, Vol. 7, Jenna M. Ziebell and Maria Cristina Morganti-Kossmann. Involvement of Pro-and Anti-Inflammatory Cytokines and Chemokines in the Pathophysiology of Traumatic Brain Injury, 22-30, 2010, with permission from Elsevier.”

1.2 Role of Cytokines during Neuroinflammation

Cytokines are small (between 10- 60kDa) secreted proteins that play a variety of roles in mediating immunity and inflammation. They can be secreted from immune cells such as monocytes, macrophages, and leukocytes and can cross the BBB. They can also be produced in the CNS by astrocytes, microglia, and neurons (6). Normally, cytokines are expressed at low levels in healthy tissue. However, they are rapidly released upon stress from their environment (7). Cytokines are stored intracellularly as precursor proteins and have multiple effects on different cell types (8-9). In response to injury, cytokines are released and may have either pro- or anti-inflammatory properties. The pro-inflammatory cytokines are often upregulated and induce the synthesis of anti-inflammatory cytokines (10). Cytokines can induce signaling by binding to specific receptors on the surface of their target cells. This can result in activation of signal transduction pathways which activates gene transcriptions that are involved in cellular processes such as cell growth and differentiation. As a result, expression levels of the cytokine receptor and signal transduction pathways of the target cell determine the cellular response to a particular cytokine. Examples of cytokines involved in neuroinflammation are the tumor necrosis factor (TNF) and interleukin (IL) family of peptides as well as transforming growth factor (TGF)- β (11-12).

Some of the major pro-inflammatory cytokines released after injury include IL-1 α , IL-1 β , IL-6, interferon- γ , and TNF- α . These proinflammatory molecules trigger inflammation through the release of chemokines, upregulation of cellular adhesion molecules, and activation of other inflammatory molecules. In contrast, another group of cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)- β are known to counteract and suppress inflammation (13). Experimental studies have shown that pro-inflammatory cytokines such as IL-1 β were found elevated in the cerebral spinal fluid and brain parenchyma of rodents as well

as patients that have undergone brain trauma. It was shown that neuronal damage was suppressed when the Interleukin-1 receptor antagonist (IL-1ra), was injected in following brain injury (14).

Anti-inflammatory cytokines are capable of suppressing the intensity of the inflammatory cascade by inhibiting the production of pro-inflammatory cytokines (15). Experimental studies have demonstrated that exogenous administration of the anti-inflammatory cytokine IL-10 “reduced pro-inflammatory cytokine expression thereby aiding neurological recovery” (16). As a result, a “balance” between the effects of pro-inflammatory and anti-inflammatory cytokines is thought to determine injury outcome. Cytokines can also exhibit dual functions during inflammation. For example, IL-6 possesses both pro- and anti-inflammatory properties. IL-6 promotes inflammation by triggering the expression of pro-inflammatory genes, influencing chemotaxis for immune mediators to migrate to the site of injury, as well as induce BBB dysfunction. The anti-inflammatory properties of IL-6 include inhibiting the production of other pro-inflammatory cytokines such as IL-1, TNF- α , and IFN- γ while promoting the synthesis of IL-1 receptor antagonists.

INTRODUCTION II: CHEMOKINES AND CHEMOKINE RECEPTORS

2.1 The structure and function of chemokines

Chemokines are chemoattractant cytokines (~8-14 kDa) involved in the migration of immune-inflammatory cells from the vascular system to the site of injury. In the immune system, chemokines play a variety of roles such as mediating cell migration to the site of inflammation, phagocytosis, cytokine secretion as well as cell activation and proliferation (17). All chemokines have an N-terminal domain with four conserved cysteine residues that are linked by disulfide bonds. The structure also has 3 antiparallel β -pleated sheets and α -helix that lies on top of the sheets. The N-terminal region of chemokines is important for receptor

activation because alterations in this domain have been shown to affect activity (18). They are divided into four families based on the number and position of the conserved cysteine residues in the N-terminal position. The following chemokines in this family are currently identified: CXC (α chemokines), CC (β chemokines), CX3C (δ chemokine), and C (γ chemokine). The structural differences among chemokines are related to functional differences and target specificity. For example, the CC chemokines recruit blood cells such as monocytes and lymphocytes. The CXC chemokines can recruit neutrophils, T lymphocytes, B lymphocytes, and natural killer cells. Chemokines bind to cell surface receptors that belong to seven-transmembrane domain G protein coupled receptors (GPCRs) (19). A model of the binding of chemokines to their receptors has been proposed (20).

2.2 Structure of Chemokine Receptors

Chemokine receptors are named after the chemokines that they bind. Thus, the receptors are named CXCRn, CCRn, CX3CRn and XCRn. The “R” stands for receptor and “n” is a number that corresponds to the order in which the receptors were identified. Currently, there are 18 known chemokine receptors that include 6 CXC receptors (CXCR1 to CXCR6), 10 CC receptors (CCR1 to CCR10), one CX3C receptor (CX3CR1) and one XC receptor (XCR1) (21) (Fig 3). Chemokine receptors have around 340-370 amino acids and possess 25-80% homology in their amino acid sequence. Chemokine receptors are seven-transmembrane G protein coupled receptors. Their most common features include a short extracellular N-terminal domain and a cytoplasmic C-terminal tail. The C-terminal domain contains the amino acid residues, serine and threonine, which are phosphorylated upon receptor activation for signal transduction. The first and second extracellular loops of the receptor are linked by disulfide bonds. The second extracellular loop also contains a conserved sequence of 9 amino acids (DRYLAIVHA) that is important for G protein interaction. Figure 4 shows a representative figure of a chemokine

receptor, CCR5. Similar to cytokines, chemokines can also bind to more than one receptor (22).
Currently, little is known about the functional consequences of this promiscuity.

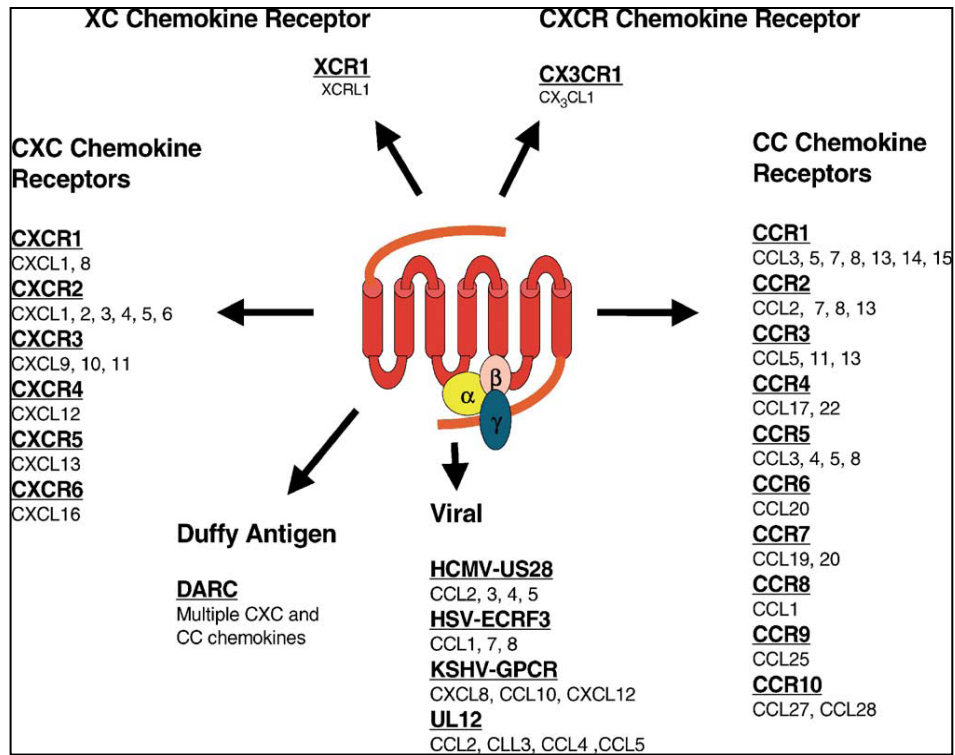


Fig 3: Chemokine Receptor Family with corresponding ligands (Fish E. 2003)

“Reprinted from Seminars in Immunology, Vol 15, Eleanor Fish, Introduction, 1-4, 2003, with permission from Elsevier.”

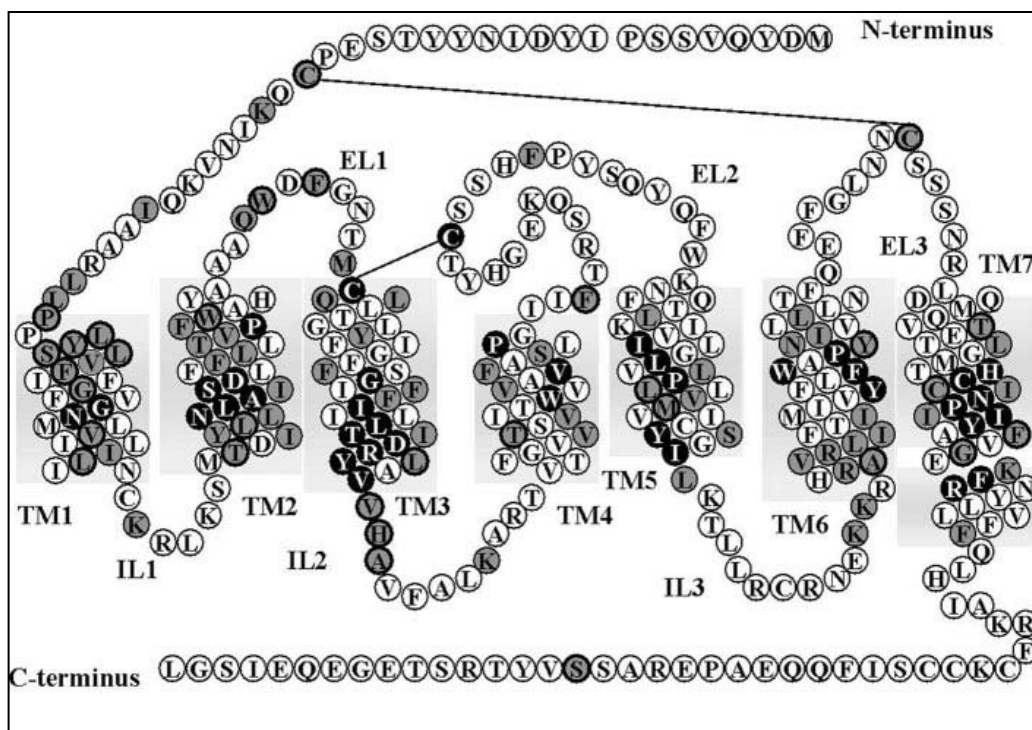


Figure 4: “Schematic representation of the CCR5 sequence. Gray rectangles outline residues in the 7-TM region, TM1 through TM7, and helix 8. EL and IL denote extracellular and intracellular loop regions, respectively. Disulfide bridges between C20 and C269 and C101 and C178 are shown as lines connecting these cysteines. Gray circles denote conservation of strong groups in CCR1 through CCR5. Gray circles with heavy outlines denote identical residues in CCR1 through CCR5. Black circles denote highly conserved residues in the rhodopsin family of GPCR. For ease of comparison with other GPCR, residues are numbered using the highly conserved residues as reference (Ballesteros and Weinstein, 1995). N:1.50 (TM1); D: 2.50 (TM2); R: 3.50 (TM3); W: 4.50 (TM4); P: 5.50 (TM5); P: 6.50 (TM6); P: 7.50 (TM7).” (Paterlini 2002) (23).

“Reprinted from Biophysical Journal, Vol 83, M. Germana Paterlini, Structure Modeling of the Chemokine Receptor CCR5: Implications for Ligand Binding and Selectivity, 3012-3031, 2002, with permission from Elsevier.”

2.3 Chemokine Receptor Signaling

Receptor signaling after chemokine activation results in the activation of heterotrimeric G-proteins ($\alpha\beta\gamma$) that are bound to the intracellular loops of the receptor (24). Specifically, the $G\alpha$ subunit has a GTPase domain that binds and hydrolyzes GTP. In the inactive state, the $G\alpha$ subunit binds to GDP. Upon ligand binding to GPCR, the receptor undergoes a conformational change in which the heterotrimeric G-protein becomes activated. This activation leads to the dissociation of GDP from the $G\alpha$ subunit and GDP is exchanged for GTP. As a result, the $G\alpha$ -GTP dissociates from the receptor leaving the $G\beta\gamma$ heterodimer. Both the $G\alpha$ -GTP and the $G\beta\gamma$ heterodimer activate different signaling pathways that eventually result in a physiological response. For example, the $G\alpha$ subunit activates Src tyrosine kinase which can activate MAP kinases, PI3 kinase, and FAK (focal adhesion kinase) (25). The release of $\beta\gamma$ subunits lead to the activation of phosphatidyl inositol specific phospholipase C (PLC) which generates diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). This activation triggers the release of calcium that can activate various protein kinase C (PKC) enzymes. The elevation of calcium is a well known response to chemokine receptor stimulation. The release of $\beta\gamma$ subunits also leads to activation of phosphatidyl inositol-3-OH kinase (PI3K) which results in the activation of the protein kinase Akt/PkB (26). The JAK/STAT pathway is also activated upon chemokine binding to the receptor (27). Figure 5 shows the signaling pathways that are activated upon chemokine binding to its receptor.

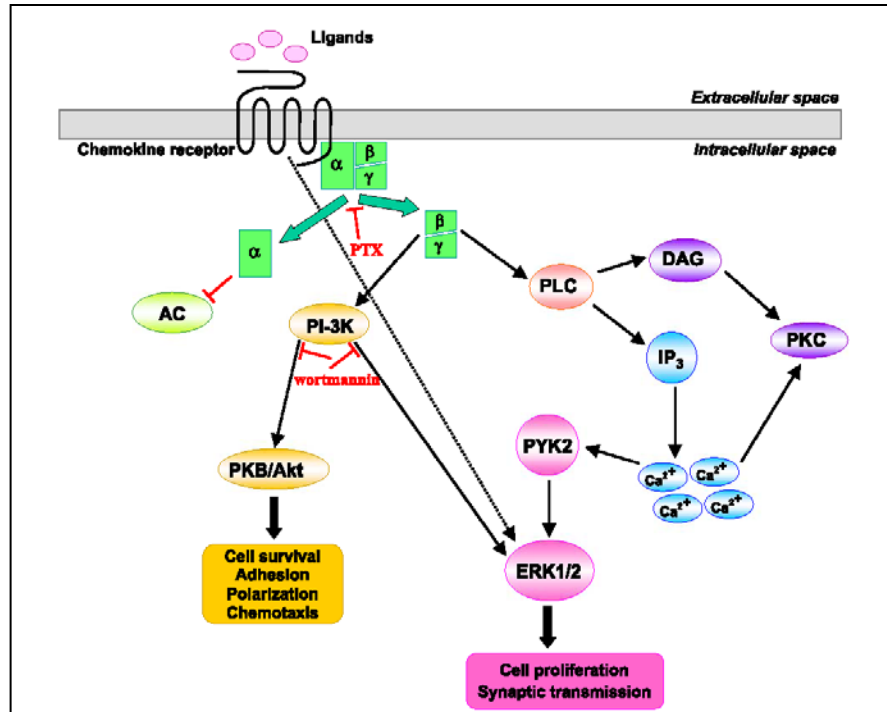


Figure 5: “Signaling pathways downstream of chemokine receptor activation. The major molecules involved in these pathways are represented, together with the cellular effects they promote. AC, adenylyl cyclase; DAG, diacylglycerol; ERK 1/2 , extracellular signal-regulated kinase; IP3, inositol triphosphate; PI-3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; PYK2, proline-rich tyrosine kinase 2.” (Cartier et al. 2005) (22)

“Reprinted from Brain Research Reviews, Vol 48, Laetitia Cartier, Oliver Hartley, Michel Dubois-Dauphin, and Kari-Heinz Krauz, Chemokine Receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases, 16-42, 2005, with permission from Elsevier.”

INTRO III: INTRODUCTION OF CCR5

3.1 CC-Chemokine Receptor 5 (CCR5)

The CC chemokine receptor 5 (CCR5) is expressed in the immune system where it is found in monocytes, leukocytes, memory T cells, and immature dendritic cells (28). CCR5 has a molecular mass of 40.6 kDa and is highly related to CCR2 (76% sequence identity). CCR5 binds to the CC- chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), and CCL8 (MCP-2) (29). Besides exhibiting characteristics of a typical G-protein coupled receptor (see Fig 6), the amino-terminal domain of CCR5 contains tyrosine residues that are posttranslationally modified by sulfation. This modification contributes to the high affinity binding of the chemokine to the receptor. In addition to the sulfation of tyrosine residues in the amino-terminal domain of CCR5, CCR5 is also modified by O-linked glycosylation (30). Chemokine binding to CCR5 is disrupted when O-linked oligosaccharides are removed (31). The carboxyl-terminus of CCR5 is palmitoylated. Cysteine residues of CCR5 at amino acid position 321, 323, and 324 are all palmitoylated and this modification may affect different functions of the receptor such as signaling, membrane targeting, endocytosis, and recycling (32). Upon binding to these ligands, CCR5 functions in the chemotaxis of immune cells such as leukocytes to areas of inflammation (33).

3.2 CCR5 serves as co-receptor for HIV

In addition to its role as a chemokine receptor, CCR5 is a co-receptor that enables cellular HIV entry (34). On the target cell, the cell surface expression of CD4 serves as a docking site for the viral envelope glycoprotein gp120 of HIV-1. The viral gp120 undergoes a conformational change once it binds to CD4 that allows gp120 to bind to CCR5 which allows the virus to enter the host cell. Specifically, CCR5 is the co-receptor required for the M-trophic

strain of HIV-1 to enter its target cell (35). The evidence that CCR5 plays a role in HIV entry began with the demonstration that the chemokines that bind to CCR5 inhibited HIV infection (36). Upon chemokine binding to CCR5, the receptor internalizes which inhibits HIV from binding to CCR5 on the cell surface to allow the virus to enter its target cell. Moreover individuals with mutation in the CCR5 gene are resistant to HIV-1 infection (37-38). For example, mutant CCR5 with a 32-bp deletion in exon 4 (CCR5 Δ 32) resulted in intracellular expression of the receptor instead of membrane expression. As a result, individuals with this CCR5 mutation were found less resistant to HIV infection. Therefore, understanding the trafficking of CCR5 may contribute to the mechanism in which chemokines protect cells from HIV-infection.

3.3 Trafficking of CCR5

The inactivation of CCR5 signaling involves its removal from the plasma membrane by endocytosis. Endocytosis is a process in which extracellular molecules are internalized and are either degraded within lysosomal compartments or recycled to the cell surface. As a result, the number of receptors on the cell membrane is dependent upon the balance between the rate of internalization and the rate of recycling. There are two major pathways by which chemokine receptors can undergo internalization after ligand binding. The first route involves binding of the protein, β -arrestin to the receptor which leads to a movement of the receptor to clathrin-coated pits and internalization. For example, when chemokine CCL5 binds to CCR5, CCR5 dimerizes and is activated in which the C-terminal serine residues of CCR5 become phosphorylated. β -arrestin 2 binds to the phosphorylated C-terminal serine residues of CCR5 thereby initiating endocytosis through clathrin-coated pits (39). Another pathway in which GPCRs are internalized involves the caveolae. This pathway is independent of clathrin-coated pits. Caveolae are small invaginations that are present in the plasma membrane. The lipid

composition of caveolae is enriched with cholesterol, sphingolipids, and glycolipids. They are involved in a variety of cellular functions such as endocytosis, photocytosis, calcium signaling, and cholesterol transport (40).

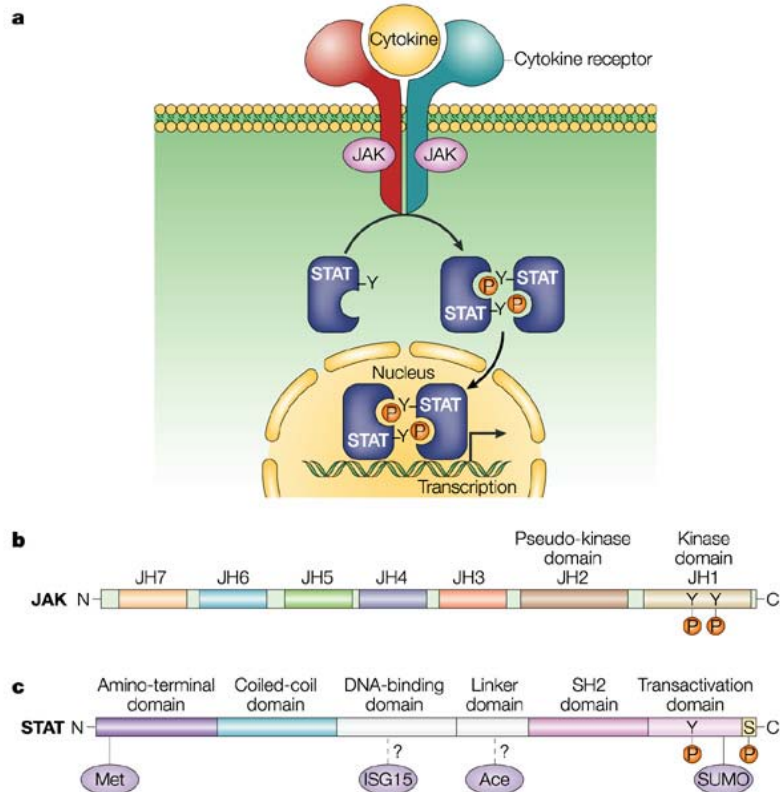
The clathrin-mediated endocytic pathway is a well known pathway in which chemokine receptors undergo internalization upon ligand binding. Experimental studies have demonstrated that agonist-induced endocytosis of CCR5 is clathrin-dependent. For example, the RNAi knock down expression of the clathrin-heavy chain showed that CCL5 induced CCR5 internalization was inhibited (41). This same study also observed that upon ligand binding, CCR5 internalized in clathrin-coated vesicles and accumulate in recycling endosomes where upon removal of the ligand, CCR5 is recycled back to the plasma membrane in a dephosphorylated form (42-43). It was also discovered that the last four amino in the C-terminal domain of CCR5 is required for the receptor to recycle back to the cell surface. In the absence of this sequence, CCR5 mutants are re-routed towards the lysosomes for degradation (44).

The internalization of CCR5 may also involve the clathrin-independent caveolae pathway. It's been found that CCR5 deficient in phosphorylation (which lack the β -arrestin protein needed for the recruitment of clathrin coated pits for endocytosis) still underwent internalization but at a slower rate compared to clathrin-dependent pathways. It was also suggested that the palmitoylated carboxyl-terminal cysteine residues of CCR5 may be involved in the association of the receptor to caveolae for internalization (45). For example, experimental studies showed that through the use of the inhibitors Filipin and Nystatin (which flattens caveolae), the internalization of CCR5 was inhibited. The use of other inhibitors in the study such as cycloheximide (prevents protein synthesis), Brefeldin A (inhibits the formation of vesicles from the Golgi), and Monensin (blocks Golgi transport) demonstrated that the trafficking of CCR5 does not involve protein synthesis or the Golgi apparatus (46).

3.4 CCR5 Signaling

CCR5 can activate multiple signal transduction pathways upon the binding of its ligands. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway have been implicated in chemokine signaling. When chemokines bind to their seven transmembrane G-protein coupled receptor, the JAK/STAT pathway is activated and results in chemotactic responses. JAK activation triggers cell proliferation, migration, differentiation, and apoptosis (47). Members of the JAK family include JAK 1, JAK 2, JAK 3, and Tyk 2 whose activation is dependent upon the cytokine/chemokine that is bound to the receptor (48). Although specific JAK proteins are activated upon the cytokine/chemokines binding to the receptor, the specificity of cytokine signaling are not determined by the JAK kinases in that many different cytokines trigger the activation of the same JAKs. Once the ligand binds to its chemokine receptor, the receptor dimerizes and undergoes a conformational change that exposes the cytoplasmic domain of the receptor allowing JAK proteins to bind. The binding of JAK proteins leads to the phosphorylation of tyrosine residues of the receptor and creating docking sites for the SH2 domain of STATs to bind (49). Currently, there are seven STATs proteins that have been identified. STATs are transcription factors that translocates to the nucleus from the cytoplasm upon activation. The C-terminus of STATs has a conserved tyrosine residue that is phosphorylated by JAK proteins. Upon phosphorylation by JAKs, STATs become activated and heterodimerizes with other STAT proteins. The dimerized STATs enter the nucleus to induce gene transcription. Therefore, the JAK/STAT signaling pathway provides a direct mechanism to translate an extracellular signal into a transcription response. In the signaling of CCR5, it's been found CCL5 binding to CCR5 induces the activation and association of JAK2 and JAK3 (50). The activation of JAK2 and JAK3 by CCL5 has been shown to play a role in influencing the intracellular elevation of calcium levels in cultured

human microglia (51). In human T cell lines, CCL3 and CCL5 also induce the activation of STAT1 and STAT3 (52). Figure 6 shows the JAK/STAT signaling pathway as well as the domain structure of JAKs and STATs.



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Fig 6 “**a** | A schematic representation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription. **b** | The domain structure of JAKs. The domains JH1–JH7 are based on sequence similarity of four known JAKs. JH1 is the kinase domain, which contains two tyrosines that can be phosphorylated after ligand stimulation. JH2 is the pseudo-kinase domain. The JH6 and JH7 domains mediate the binding of JAKs to receptors. **c** | The domain structure of STATs. The activity of STATs can be regulated by protein modification, including tyrosine and serine phosphorylation, methylation (Met), sumoylation (SUMO), ISGylation (ISG15) and acetylation (Ace). The modification sites of ISGylation and acetylation have not been identified.” (Shuai K. and Liu B. 2003) (53).

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In addition to the JAK/STAT pathway, CCL4 and CCL5 activate the Akt/PKB pathway upon binding to CCR5. This pathway was found to mediate neuronal protection against HIV/gp120 neurotoxicity (54). It is known that HIV-1 can cause neurological problems. Although the virus primarily infects macrophages or microglia, neuronal damage may also occur as a result of the infection. (55). Experimental studies using mouse cerebrocortical cultures have demonstrated that CCR5 may mediate neuronal survival from HIV-1/gp120 through the activation of the Akt/PKB pathway and that inhibition of AKT disrupted neuronal protection. Previous studies have also shown that AKT was reported to protect against excitotoxic neuronal death (56). AKT/PKB is a serine/threonine protein kinase that plays multiple roles in the regulation of cell survival, proliferation, adhesion, polarization, and chemotaxis (57). Upon chemokine binding to its receptor, G proteins dissociate and the G $\beta\gamma$ subunit may activate PI3-kinase which activates AKT/PKB. The activation of AKT/PKB mediates cellular survival by inhibiting apoptosis. For example, one of the downstream targets of AKT is a pro-apoptotic member of the Bcl-2 family named BAD. BAD is known to induce apoptosis through the release of cytochrome C from the mitochondria which leads to activation of Caspases that leads to cellular death. However, when AKT phosphorylates Ser136 of BAD, its pro-apoptotic activity is inhibited (58). Similarly, Caspase-9 also plays a role in initiating apoptosis. When AKT phosphorylate serine residue 196 of Caspase-9, its activities are also diminished (59). In addition, AKT is also known to regulate cellular survival through transcription factors that regulate pro- as well as anti-apoptotic genes. Other pathways that are activated upon CCL4 and CCL5 binding to CCR5 is the activation of ERK and the Lyn/PI3K/Pyk2 signaling complex that has been shown to be important for regulating chemotaxis in primary human macrophages (60).

INTRO IV: Chemokines and Chemokine receptors in the CNS

In the central nervous system, chemokines and their receptors are expressed by astrocytes, microglia, endothelial cells, oligodendrocytes, and neurons (61). They are involved in the brain's response to injury and infection. Similar to cytokines, the expression of chemokines in normal brain tissue occurs at low levels. However, after brain injury, chemokine levels are found elevated. For example, following brain injury in mice, chemokine mRNA levels were upregulated compared to normal, uninjured mice (62). In a clinical study of patients with posttraumatic brain contusions, the mRNA levels of chemokines such as CCL2, CCL3, CCL4, CCL5, and CCL8 were elevated (63). The upregulation of chemokines in the brain after injury may be the result of the activation of astrocytes and microglial cells that secrete chemokines in response to the injury. In addition to chemokines, experimental studies have also shown that the mRNA and protein levels of chemokine receptors were also found elevated following injury. For example, in a model of excitotoxic brain injury, CCR5 mRNA levels were observed to be upregulated compared to normal, uninjured animals. The presence of upregulated chemokine receptors such as CCR5 after brain injury may be due to the elevated levels of chemokines in response to injury.

4.1 CCR5 in Brain Development

Chemokines and their receptors have been shown to participate in the development of the CNS during embryogenesis. For example, it has been shown that the chemokine receptors CCR5 and CXCR4 are detected in primary cultures of human embryonic neurons (64). The expression of CCR5 and CXCR4 were also observed in resident cells of the cortex, hippocampus, thalamus, cerebellum, and brain stem from embryonic to adult Rhesus Monkey. CCR5 and CXCR4 were expressed on neurons and glia during development and the expression levels of both chemokine receptors increased in the neocortex with age. For example, in the

third-trimester fetus, CCR5 appeared to be weakly immunopositive in scattered cortical neurons. At birth, there was still low expression of CCR5 in most of the pyramidal neurons of the cortex and hippocampus. However, between 3 and 14 days of age, the expression of CCR5 appeared to increase and by 50 days of age, the expression levels of CCR5 were comparable to protein levels of CCR5 found in adult by 9 months of age. The early onset and rapid increase of CCR5 expression from fetus to adult brains in Rhesus monkey suggests that CCR5 may play a role in brain development.

The presence of chemokines and their receptors during CNS development has been implicated to play roles in directing the migration of immature neurons as well as precursor glial cells to their destination site within different regions of the developing brain. Because chemokines are known to guide peripheral immune cells such as leukocytes to the site of inflammation, it may be possible that their mechanisms of action are recapitulated during brain development. For example, chemokines and their receptors have been found to be involved in the development of the cerebellum and hippocampus. CXCR4 is a chemokine receptor that has similar functions to CCR5 in that it is also a co-receptor required for HIV to bind in order for the virus enter the host cell (65). Experimental studies showed that during cerebellar development, CXCR4 knock-out mice resulted in disruption of the migration of granule cells (tiny neurons) to form the internal granule layer (IGL) that is required for proper cerebellum development (66). In addition to the cerebellum, CXCR4 knock-out mice also resulted in the improper formation of the granule layer of the dentate gyrus in the hippocampus (67). These data suggests that chemokines and their receptors may play a role in neuronal migration during brain development.

4.2 CCR5 expression in the CNS

CCR5 receptors are expressed in normal and diseased and injured brain. CCR5 and its ligands are expressed in microglia, astrocytes, neurons, and endothelial cells however, the preponderance of evidence links CCR5 with activated microglial cells suggesting a role in post injury repair. CCR5 is found on activated microglial cells in Multiple Sclerosis (68) and Alzheimer's disease (69). The ligands of CCR5 are upregulated in a rat model of stab wound brain injury (70). The expression of CCR5 mRNA is also elevated after excitotoxic brain injury in neonatal rats (71-72) as well as in hypoxia induced brain injury in rats (73). CCR5 plays a role in microglial migration towards the lesion site after focal brain injury (74). In addition, CCR5 and its ligands were found to be expressed during brain development suggesting potential roles in regulating CNS development (75-76).

In this study, we examined the expression and localization of CCR5 in the rat brain before and after traumatic brain injury. The time course and cellular localization of CCR5 are described and reveal that the receptor is upregulated in a time-dependent manner in neurons of the parietal cortex and hippocampus. The absence of CCR5 expression in microglia and its presence in neurons after injury suggests a role for CCR5 in neuronal survival after injury.

MATERIALS AND METHODS

5.1 Animals

All experimental procedures were approved by the *Institutional Animal care and Use Committee* in compliance with the National Institutes of Health guidelines outlined in *Guide for the Care and Use of Laboratory Animals*. Male Sprague Dawley rats (300g) were purchased from Harlan (Indianapolis, IN).

5.2 Antibodies

CCR5 (sc-17833), MAP-2 (sc-20172), GFAP (sc-6171), HA-probe (sc-7392) were all purchased (Santa Cruz Biotech, Santa Cruz, CA). Iba-1 was purchased (Wako Chemicals, Richmond, VA). FLAG was purchased (Stratagene, La Jolla, CA). The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Anti-human CCR5 MAb (Clone 45531, Cat# 4087) from DAIDS, NIAID.

5.3 Controlled cortical impact injury

A controlled cortical impact device (CCI) was used to initiate a unilateral brain injury as described previously (77). The procedure was performed as previously described (78-79). Rats were anesthetized using 4% isoflurane with a 2:1 mixture of N₂O/O₂ and then maintained with a 2% isoflurane and 2:1 N₂O/O₂ mixture via a face mask. Animals were placed in a stereotaxic frame and a 7 mm craniotomy (halfway between bregma and lambda, 3.5mm lateral to midline) was performed. In order to maintain the animals' body temperature at a constant 37° , a heating pad was used. Using a 6 mm diameter impact tip, a single impact (2.7 mm deformation, 6 m/s) was delivered on the right parietal lobe at an angle of 10 degrees from the vertical plane, such that the impact was orthogonal to the cortex surface. These parameters

produce a moderate to severe injury. Sham-operated animals received all surgical procedures except the impact were used as controls.

5.4 Sample Preparation

At various time points following injury, animals were sacrificed and brains were dissected and submerged under ice-cold artificial cerebrospinal fluid (10 mM HEPES pH 7.2, 1.3 mM NaH₂PO₄, 3 mM KCl, 124 mM NaCl, 10 mM dextrose, 26 mM NaHCO₃, and 2 mM MgCl₂). The penumbra cortex and hippocampi were quickly removed and snap-frozen on dry ice. The tissues were homogenized in a lysis-buffer containing 10 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 μ M dithiothreitol (DTT), 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 μ M okadaic acid (80).

5.5 Western Analysis

Protein concentrations for each sample were measured using BCA protein assay kit (Pierce, St. Louis, Mo.) according to the manufacturers' instruction using bovine serum albumen (BSA) as a standard. Samples were resolved on SDS-polyacrylamide gels (12% acrylamide) and transferred to nitrocellulose. Blots were stained with Ponceau S to ensure accuracy of protein loading, blocked in PBS with 5% dry milk and incubated with anti-mouse CCR5 (0.2 μ g/ml; Santa Cruz Biotech., Santa Cruz, CA.) overnight at 4°C. Membranes were washed and antibody labeling was visualized on x-ray film using HRP-conjugated secondary antibody and chemiluminescence (Pierce Chemical Co., Rockford, IL).

5.6 Immunohistochemistry

At various time points following injury, rats were anesthetized and perfused with 4% paraformaldehyde. Brains were removed, cryoprotected in 30% sucrose (in PBS) and sectioned into 40 μ m thick slices using a cryostat. Sections were incubated in primary antibodies diluted in immuno-blocking buffer (TBS-T with 2.5% normal goat serum, 2% bovine serum albumin and

0.25% Triton X-100) and incubated overnight at 4°C. Antibodies used for immunohistochemistry were NIH CCR5 (3µg/ml); MAP-2 (2µg/ml); GFAP (2µg/ml); and Iba-1 (0.5µg/ml). Sections were washed with 1x TBS-T five times for 10 min each. Secondary antibodies (Alexa fluor 568 goat anti-rabbit and alexa fluor 488 goat anti-mouse; Invitrogen, Carlsbad, CA) were incubated in immuno-blocking buffer at 4µg/ml at room temperature for 1 hour. After the sections were washed in 1x TBS-T five times for 10 min each, they were mounted on slides and dried at room temperature. The sections were cover-slipped with Fluoromount-G (SouthernBiotech Inc., Birmingham, AL) to retard fading. Confocal images were captured using the Zeiss LSM 510 laser scanning microscope equipped with the appropriate filter sets. Low power images were observed using 10x objectives showing the site of injury and its surroundings. High power images were captured at approximately 500µm away from the injured site using 63x objectives with immersion oil. Serial optical sections throughout the z-axis plane were taken at 63x magnification.

5.7 Statistical Analysis

Western blots of TBI time course of cortex and hippocampus tissue were analyzed using one-way ANOVA. Significant differences were determined at $P < 0.05$. The points at which differences were observed were identified by post hoc analysis using Tukey's method (significance were assigned at the 0.05 level).

5.8 Plasmids, Cell Culture Treatment, and Transfections

Human CXCR4 (HA- tagged) and Human CCR5 (FLAG-tagged) were kindly donated by Dr. Ricardo Richardson of North Carolina Central University. HeLa cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin on coverslips in 6-well plates and maintained at 37°C in a humidified

chamber. HeLa cells were transfected with 2-3 μ g of pcDNA3 containing the receptor cDNAs using Polyfect Transfection Reagent (Qiagen, Valencia, CA.) according to the manufacturer's instruction.

5.9 Immunfluorescence Microscopy (for transfection experiment)

After transfection, cells on coverslips were fixed in 2% paraformaldehyde for 15 minutes. Cells were washed with 1xPBS three times every 10 minutes. Cells were incubated in primary antibodies diluted in immuno-blocking buffer (2% normal goat serum and 0.25% saponin in 0.1mM phosphate buffer, pH 7.4) and incubated overnight at 4°C. Antibodies used were NIH CCR5 (3 μ g/ml), FLAG (20 μ g/ml), and HA-probe (2 μ g/ml). Cells were washed with 1xPBS three times for 10 minutes each. Secondary antibody (Alexa fluor 488 goat anti-mouse) were used at 4 μ g/ml in immuno-blocking buffer and incubated at 37°C for 30 minutes. Cells were washed with 1xPBS three times for 10 minutes each. Coverslips were mounted on glass slides with phenylenidiamine anti-fade medium (1mg/ml in 50% glycerol/PBS). Confocal images were captured using the Zeiss LSM 510 laser scanning microscope equipped with the appropriate filter sets.

RESULTS

6.1 Time course of CCR5 protein expression in cortex and hippocampus after TBI.

To investigate whether CCR5 expression is altered after traumatic brain injury, I examined the levels of CCR5 in cortex and hippocampus at various times after injury. A controlled cortical impact (CCI) device was used to produce brain injury in rats as previously described (please see Methods). At post-injury time points of 30 minutes, 3 hours, 1 day, 3 days, 14 days, and 28 days, rats were sacrificed and cortical tissue surrounding the injury site as well as the underlying hippocampal tissue were collected. Quantitative western blot analysis of the resulting lysates revealed an increase of CCR5 expression after brain injury in cortex (Fig 7A). In comparison to sham animals, the expression of CCR5 (~46-kDa band) was increased 30 minutes, 3 hours, and 1 day after injury. Interestingly three days after injury, CCR5 expression levels were not significantly different than sham controls. However, CCR5 levels were significantly increased at 14 and 28 days after injury. Statistical analysis using one-way ANOVA revealed significant differences in CCR5 protein levels post-injury ($p < 0.05$). CCR5 protein levels were significantly different from sham controls at 30 minutes, 3 hours, 14 days, and 28 days (Fig 7B) post-injury. This data suggest that after brain injury, CCR5 levels are increased at both early and later times perhaps in response to the inflammatory events that occur during these stages.

I also examined the time course of CCR5 expression levels in the hippocampus underlying the cortical injury. Quantitative western analysis revealed an increase in CCR5 levels over time in hippocampus (Fig 8A). CCR5 protein levels steadily increase over time with significant increases 14 days and 28 days after injury. Statistical analysis using one-way ANOVA revealed significant differences in CCR5 protein levels post-injury ($p < 0.05$). CCR5

protein levels were significantly different from sham controls 14 and 28 days after injury (Tukey's test $p < 0.05$) (Fig 8B). This data suggest that after TBI, CCR5 protein levels are significantly increased in the hippocampus in a time-dependent manner.

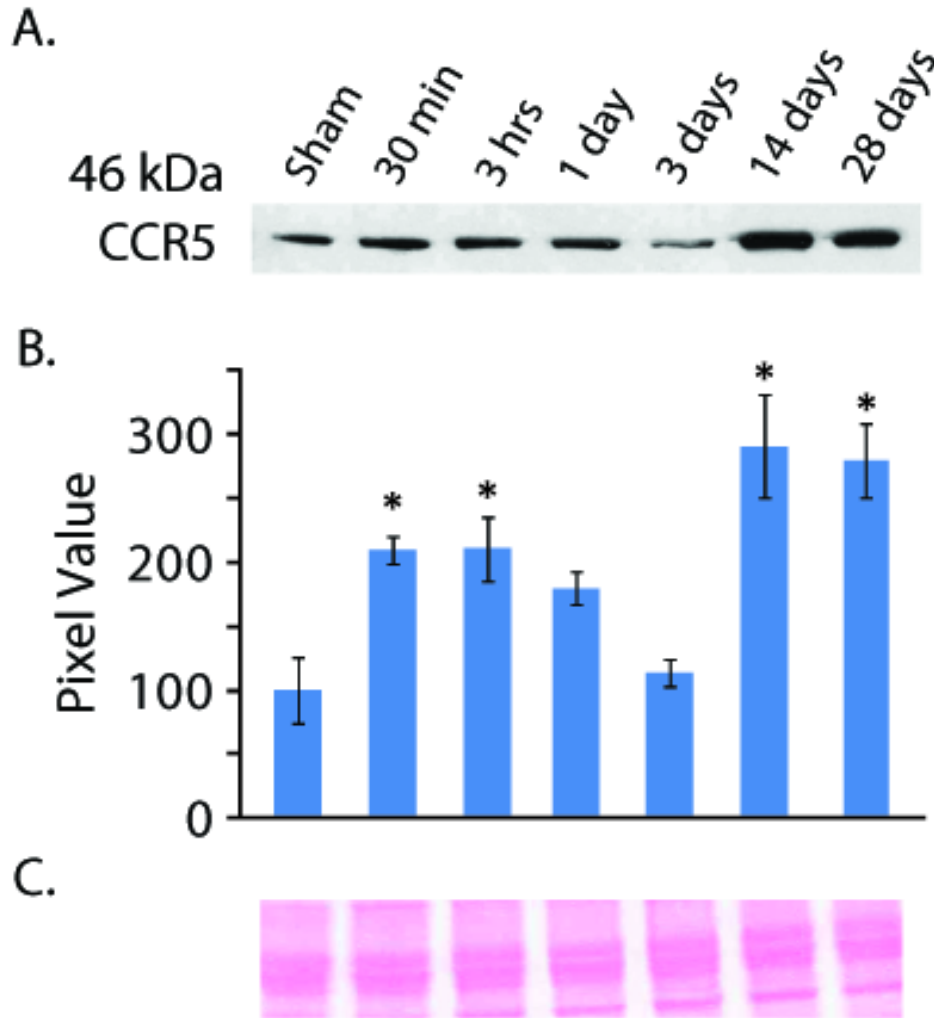


Fig 7. Time course of CCR5 protein expression levels in cerebral cortex after TBI. A. Western blot analysis of CCR5 protein expression in cerebral cortex surrounding the injury site. At various times following injury, samples were collected as described in Methods. Samples (30 μ g) were separated electrophoretically and transferred onto nitrocellulose. Membranes were probed with CCR5 antibodies and visualized using chemiluminescence and resulting bands were analyzed using NIH Image J. **B.** Quantitation of CCR5 levels in cortex from western blot analysis (mean \pm S.E.M.). (*) represents significant differences compared to Sham injured cortex (control, ANOVA with post hoc Tukeys test). **C.** Ponceau stain of transferred samples to indicate equal loading.

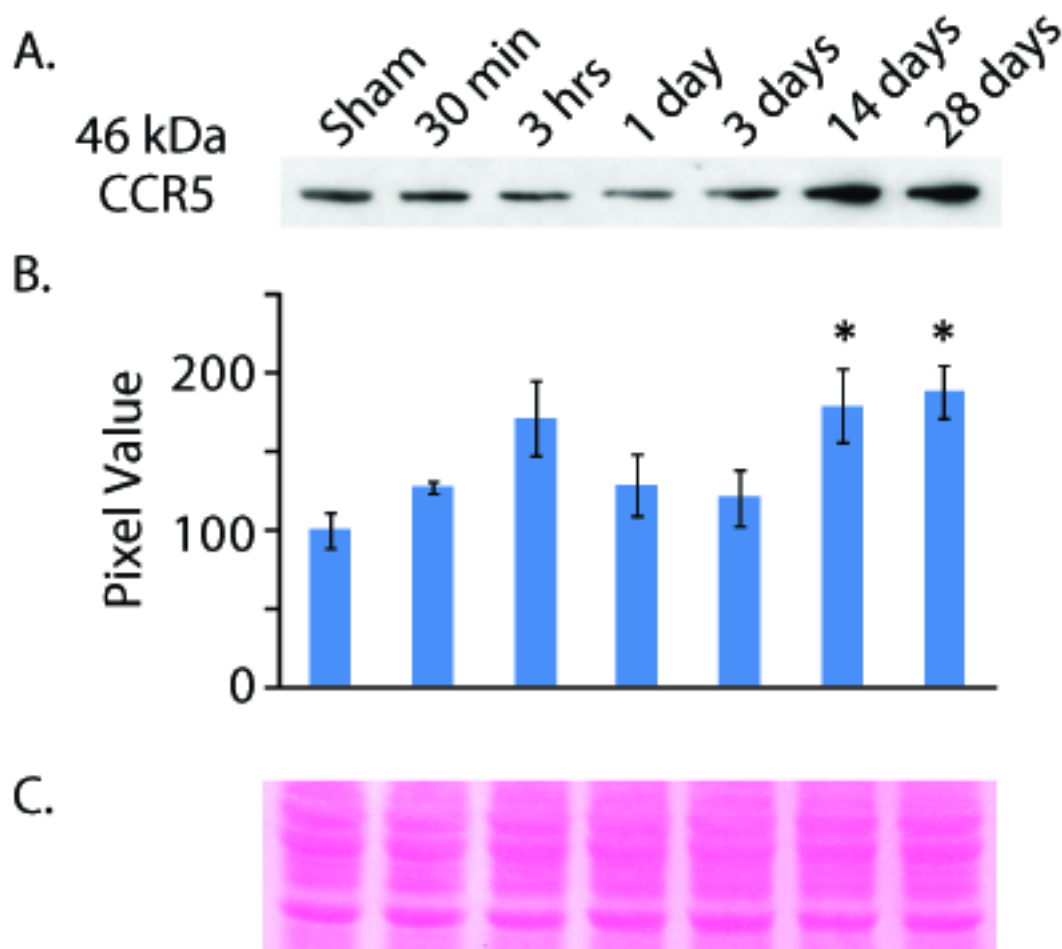


Fig 8. Time course of CCR5 protein expression levels in hippocampus after TBI. Western blot analysis of CCR5 protein expression in hippocampus underneath the injury site. At various times following injury, samples were collected as described in Methods. Samples (50µg) were separated electrophoretically and transferred onto nitrocellulose. Membranes were probed with CCR5 antibody and visualized using chemiluminescence and resulting bands were analyzed using NIH Image J. **B.** Quantitation of CCR5 levels in hippocampus from western blot analysis (mean \pm S.E.M.). (*) represents significant differences compared to Sham (control, ANOVA with post hoc Tukeys test). **C.** Ponceau stain of transferred samples to indicate equal loading.

6.2 CCR5 is localized in neurons in cortex after TBI

We wanted to determine in which cell types the increased CCR5 was localized. Based on my western analysis of the time course of CCR5 expression after injury in cortex, I selected 3 hours, 3 days, and 28 days to examine CCR5 localization. Using immunofluorescence with specific antibodies, we investigated the expression of CCR5 around the site of injury in cortex. The area of analysis of CCR5 localization is depicted with the red rectangle shown (Fig 9A). This area represents the site of injury in cortex and expression of CCR5 was observed surrounding the injury site. Figure 9 (B-E) shows the relative expression of CCR5 surrounding the site of injury. The fluorescent signal indicating CCR5 protein expression of CCR5 appeared to be stronger at areas within 500 μm of the lesion site. Therefore, I examined the cellular expression of CCR5 at a distance of 500 μm from the injury site (white box). In the sham control (Fig 9F), there is generally lower expression of CCR5 compared to all other time points (Fig 9G-I). CCR5 appeared to be expressed in cell bodies of large cells at all cortical levels (Fig 9F-I). To examine in which cells CCR5 resides, I co-labeled with MAP-2 (Fig 9J-M). MAP-2 is a microtubule-associated protein of brain tissue and is specifically expressed in the cell body and dendrites of neurons. Examination of the expression of CCR5 and MAP-2 revealed co-localization of these markers suggesting that after brain injury, CCR5 is localized in neurons (Fig 9N-Q).

Figure 9

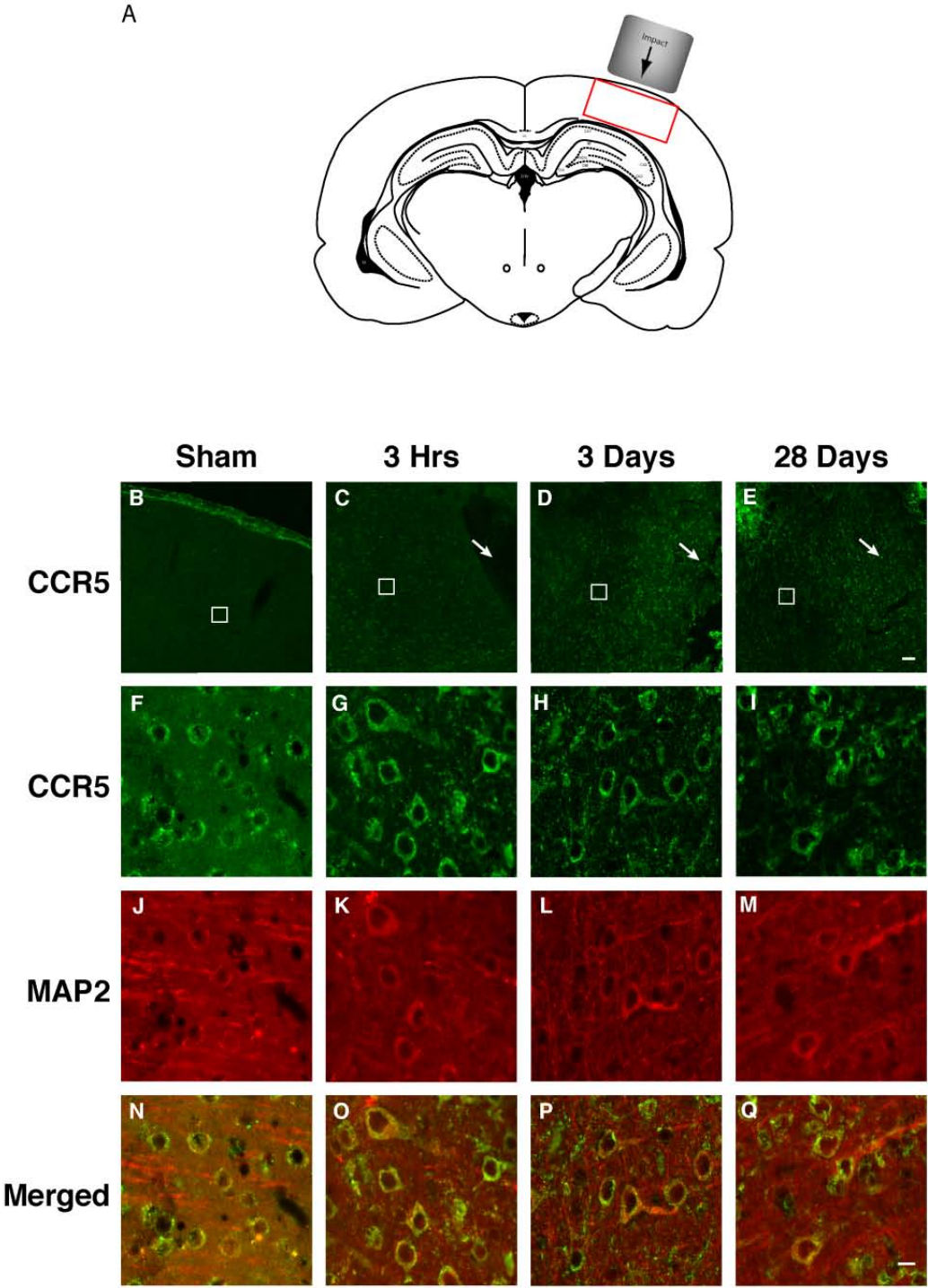


Fig 9. Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI. Free-floating tissue sections were labeled with antibodies against CCR5 (green) and MAP-2 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images **B-E** indicate regions where high power images (**F-Q**) were collected. CCR5 expression was observed in sham injured (**B, F, J, N**); 3 hours post injury (**C, G, K, O**); 3 days post injury (**D, H, L, P**); and 28 days post injury (**E, I, M, Q**). Low magnification images show the localization of collected images with respect to the injury site (**B-E, white boxes**). Arrows in boxes **C-E** indicate the site of injury. Merged images of CCR5 and MAP-2 for each time point are shown (**N-Q**). Co-localized regions are represented in yellow. Scale bar in E (panels B-E)= 50 μ m and scale bar in Q (panels F-Q)= 10 μ m.

To examine whether CCR5 might also be localized in astrocytes, I labeled injured brain sections with CCR5 and glial fibrillary acid protein (GFAP). GFAP is the chief intermediate filament protein expressed by astrocytes. Sham brain sections and brain sections taken from injured 3 hours, 3 days, and 28 days prior to harvesting were double labeled with CCR5 and GFAP. The area of analysis of CCR5 localization is depicted within the red rectangle as shown (Fig 10A). Figure 10 (B-E) shows relative expression of CCR5 surrounding the site of injury. The fluorescent signal indicating CCR5 protein expression appeared to be stronger at areas within 500 μm of the lesion site. Therefore, I examined the cellular expression of CCR5 at a distance of 500 μm from the injury site (white box). Figure 10 J-M shows expression of GFAP. Examination of the expression of CCR5 and GFAP revealed that CCR5 does not appear to co-localize with GFAP suggesting that after brain injury, CCR5 is not detectable in astrocytes (Fig 10N-Q).

Figure 10

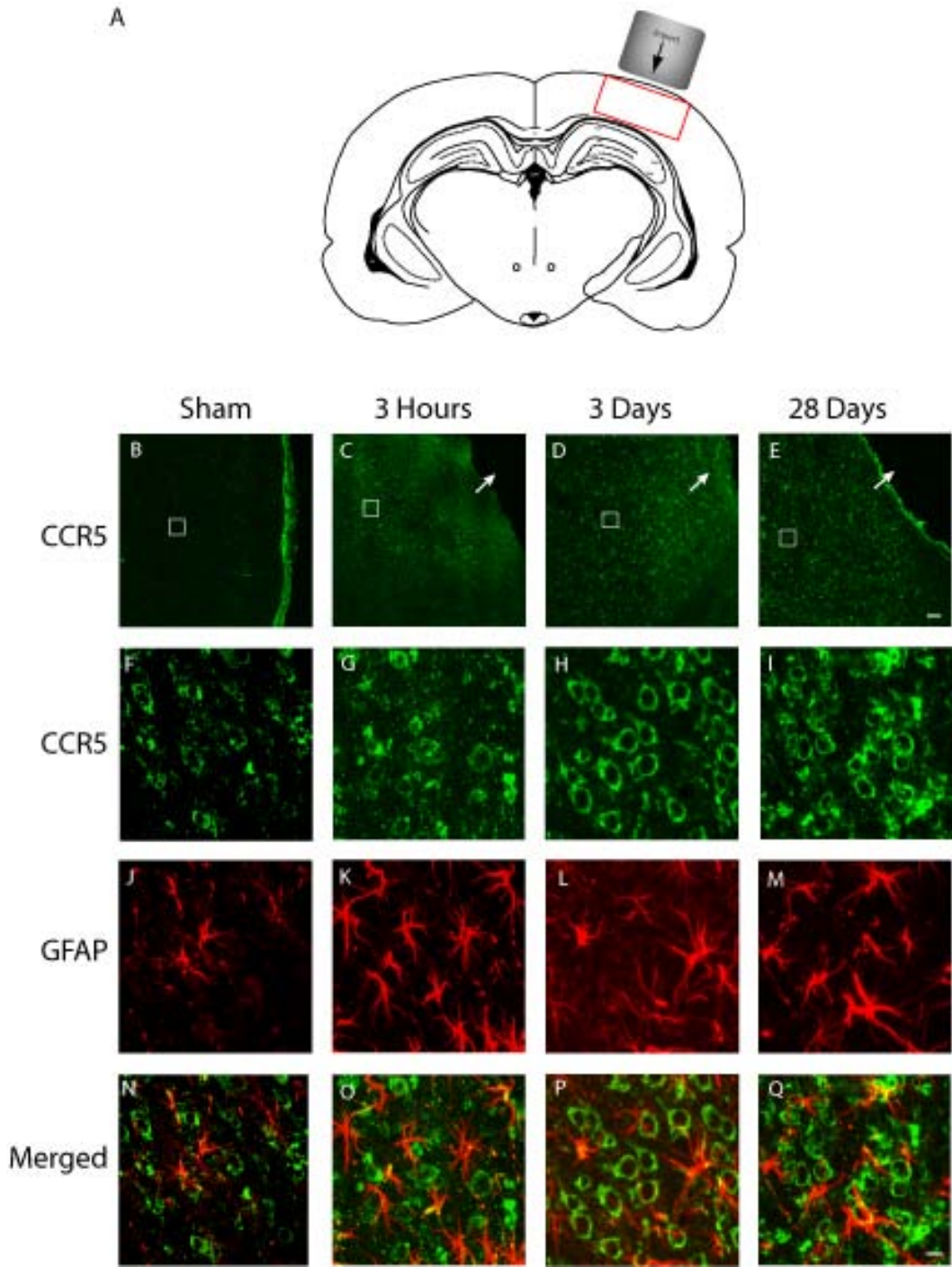


Fig 10. Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI. Free-floating tissue sections were labeled with antibodies against CCR5 (green) and GFAP (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images **B-E** indicate regions where high power images (**F-Q**) were collected. CCR5 expression was observed in sham injured (**B, F, J, N**); 3 hours post injury (**C, G, K, O**); 3 days post injury (**D, H, L, P**); and 28 days post injury (**E, I, M, Q**). Low magnification images show the localization of collected images with respect to the injury site (**B-E, white boxes**). Arrows in boxes **C-E** indicate the site of injury. Merged images of CCR5 and GFAP for each time point are shown (**N-Q**). Co-localized regions are represented in yellow. Scale bar in E (panels B-E)= 50 μ m and scale bar in Q (panels F-Q)= 10 μ m.

To examine whether CCR5 is expressed in microglia, brain sections from sham and brain-injured rats were labeled with antibodies against CCR5 and a microglial marker, Iba-1. Iba-1 is an ionized calcium binding adaptor molecule and is specifically expressed in activated microglia/ macrophages. The area of analysis of CCR5 localization is depicted within the red rectangle shown (Fig 11A). Figure 11 (B-E) shows relative expression of CCR5 surrounding the site of injury. The fluorescent signal indicating CCR5 protein expression appeared to be stronger at areas within 500 μm of the lesion site. Therefore, I examined the cellular expression of CCR5 at a distance of 500 μm from the injury site (white box). Figure 11(J-M) shows expression of Iba-1. The microglia morphology in the sham brain section (Fig. 11J) appears ramified with numerous branching processes extending out of the cell. At 3 hours after injury, those extended branches appears to shrink which indicates that the ramified microglia has become reactive or amoeboid (Fig. 11L) after brain injury. The brain section at 3 days after injury shows a cluster of reactive microglia near the site of injury (Fig. 11M). At 28 days after injury, the microglia morphology is still at a reactive state (Fig. 11M) with very little branching processes extending from the cell. Examination of the expression of CCR5 and Iba-1 revealed that CCR5 does not appear to co-localize with Iba-1 suggesting that after brain injury, CCR5 is not detectable in microglia (fig 11N-Q).

Figure 11

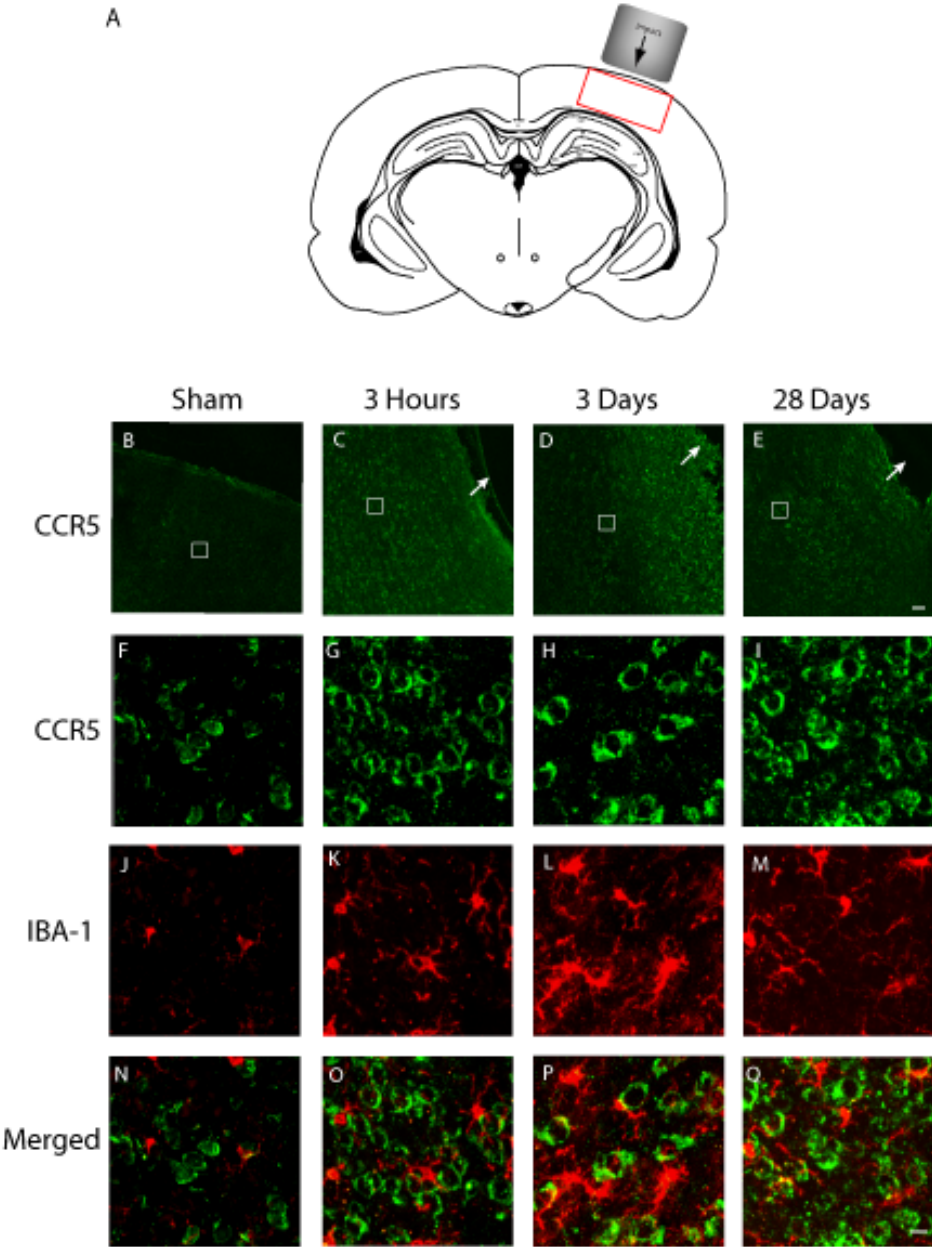


Fig 11. Immunohistochemical analysis of CCR5 protein expression and distribution in cortex after TBI. Free-floating tissue sections were labeled with antibodies against CCR5 (green) and Iba-1 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images **B-E** indicate regions where high power images (**F-Q**) were collected. CCR5 expression was observed in sham injured (**B, F, J, N**); 3 hours post injury (**C, G, K, O**); 3 days post injury (**D, H, L, P**); and 28 days post injury (**E, I, M, Q**). Low magnification images show the localization of collected images with respect to the injury site (**B-E, white boxes**). Arrows in boxes **C-E** indicate the site of injury. Merged images of CCR5 and Iba-1 for each time point are shown (**N-Q**). Co-localized regions are represented in yellow. Scale bar in E (panels B-E)= 50 μ m and scale bar in Q (panels F-Q)= 10 μ m

CCR5 is localized in neurons in hippocampus after TBI

Since CCR5 expression was increased in the hippocampal tissue underlying cortical injury, I also examined the expression of CCR5 in the hippocampus following injury to determine its cellular localization. Each figure represents images taken from sections harvested from rats at various times after TBI.

CCR5 was expressed in sham control tissue (Fig. 12). The area of analysis of CCR5 localization is depicted within the red rectangle and specifically in the CA 1/2 and CA3 regions of the hippocampus (Fig. 12A). Sham brain sections were labeled with CCR5 and MAP-2. Low power images of the CA 1/2 region (Fig 12. B-D) and CA 3 region (Fig 12. H-J) are depicted to show relative expression of CCR5 and MAP-2 across these hippocampal regions. High power images show that CCR5 was detected in the CA 1/2 region (Fig 12F) and CA 3 region (Fig 12L) of the hippocampus in sham injured rats. The merged images of CCR5 and MAP-2 in the CA 1/2 region (Fig 12G) and CA 3 region (Fig 12M) reveal co-localization suggesting that CCR5 is localized in neurons within the hippocampus.

The expression of CCR5 in hippocampal tissue taken from rats three hours post-injury in both the CA 1/2 region and CA 3 region appeared to be increased in comparison to sham control tissue (Fig 13 F,L compared to Fig 12 F,L). The three hour brain sections were labeled with CCR5 and MAP-2 and reveal co-localization of CCR5 with MAP-2 in the CA 1/2 region (Fig 13G) and in the CA3 region (Fig 13M) suggesting that CCR5 is localized in neurons 3 hours after injury.

Three days following TBI, CCR5 expression remains high in both the CA 1/2 region and CA 3 region of hippocampus compared to sham control (Fig 14 F, L compared to Fig 12 F, L). Labeling of hippocampus sections of CCR5 and MAP-2 revealed co-localization in the CA

1/2 region (Fig 14G) and in the CA3 region (Fig 14M) suggesting that CCR5 is expressed in neurons 3 days after injury.

Twenty-eight days following TBI, CCR5 expression still remains high in both the CA 1/2 region and CA3 region of hippocampus compared to sham (Fig 15 F, L compared to Fig 12 F,L). The twenty-eight days brain sections were labeled with CCR5 and MAP-2 and reveal co-localization of CCR5 with MAP-2 in the CA 1/2 region (Fig 15G) and in the CA3 region (Fig 15M) suggesting that CCR5 is localize in neurons 28 days after injury.

Figure 12

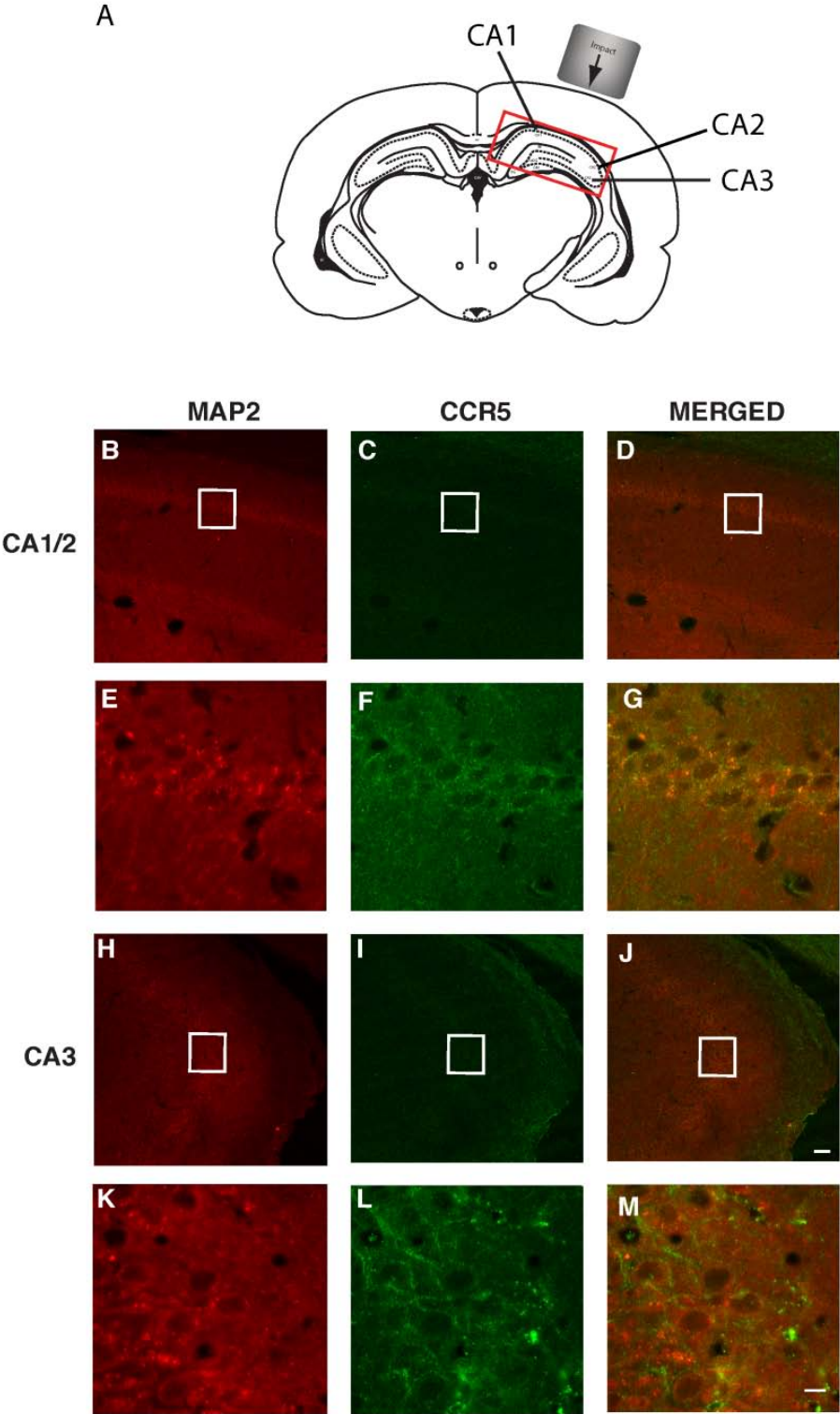


Fig 12. Immunohistochemical analysis of CCR5 protein expression in hippocampus from sham injured rat brain. Free-floating tissue sections were labeled with CCR5 (green) and MAP-2 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images (**CA1/2: B-D**, **CA3: H-J**) indicate regions where high power images (**CA1/2: E-G**, **CA3: K-M**) were collected. Scale bar in J= 50 μ m and in M= 10 μ m.

Figure 13

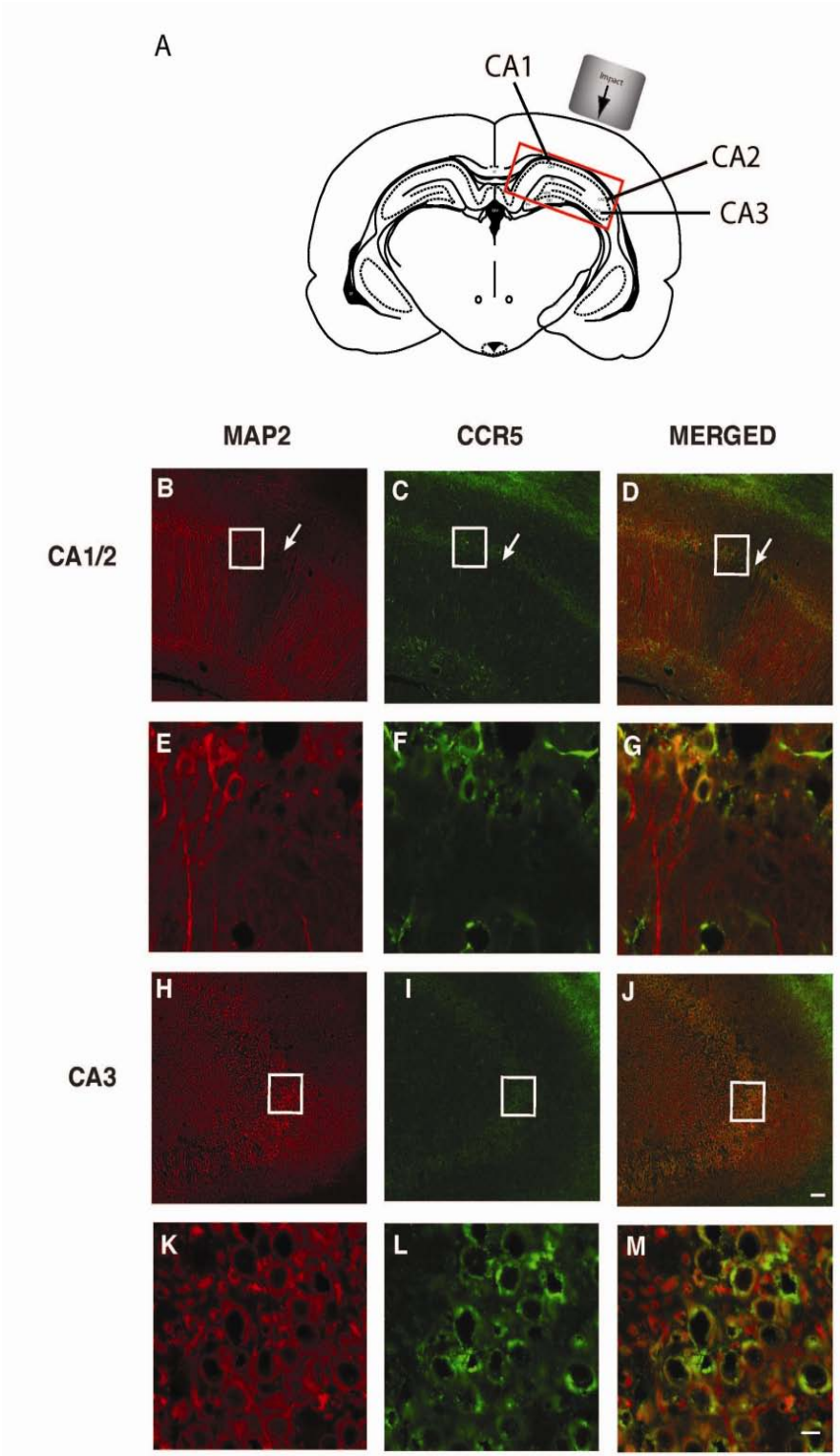


Fig 13. Immunohistochemical analysis of CCR5 protein expression in hippocampus 3 hours post- injury. Free-floating tissue sections were labeled with CCR5 (green) and MAP-2 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images (**CA1/2:B-D**, **CA3: H-J**) indicate regions where high power images (**CA1/2: E-G**, **CA3: K-M**) were collected. Arrows in low power images (**B-D**) indicate sites of injury. Scale bar in J= 50 μ m and in M= 10 μ m.

Figure 14

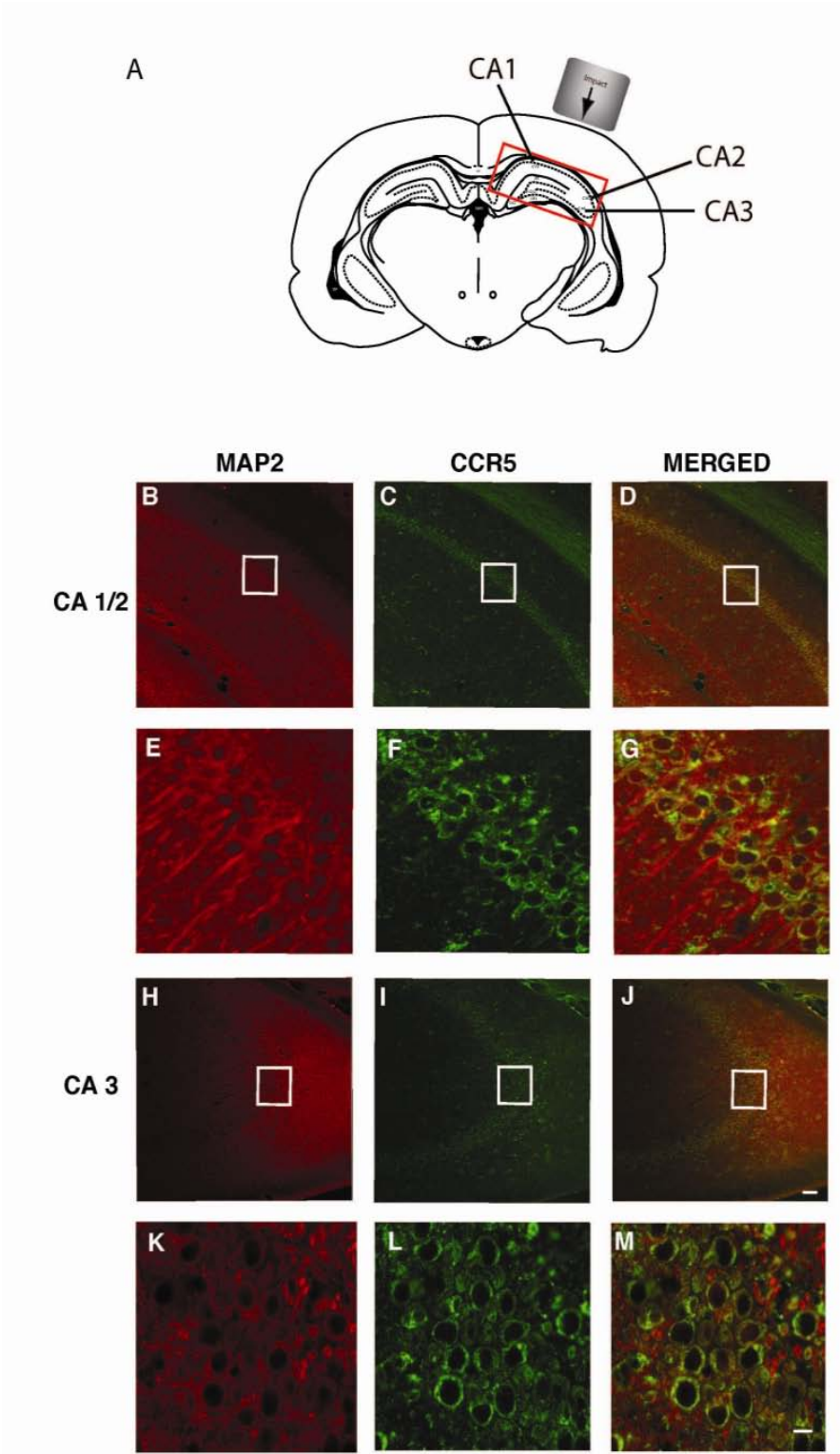


Fig 14. Immunohistochemical analysis of CCR5 protein expression in hippocampus 3 days post injury. Free-floating tissue sections were labeled with CCR5 (green) and MAP-2 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images (**CA1/2: B-D**, **CA3: H-J**) indicate regions where high power images (**CA1/2: E-G**, **CA3: K-M**) were collected. Scale bar in J= 50 μ m and in M= 10 μ m.

Figure 15

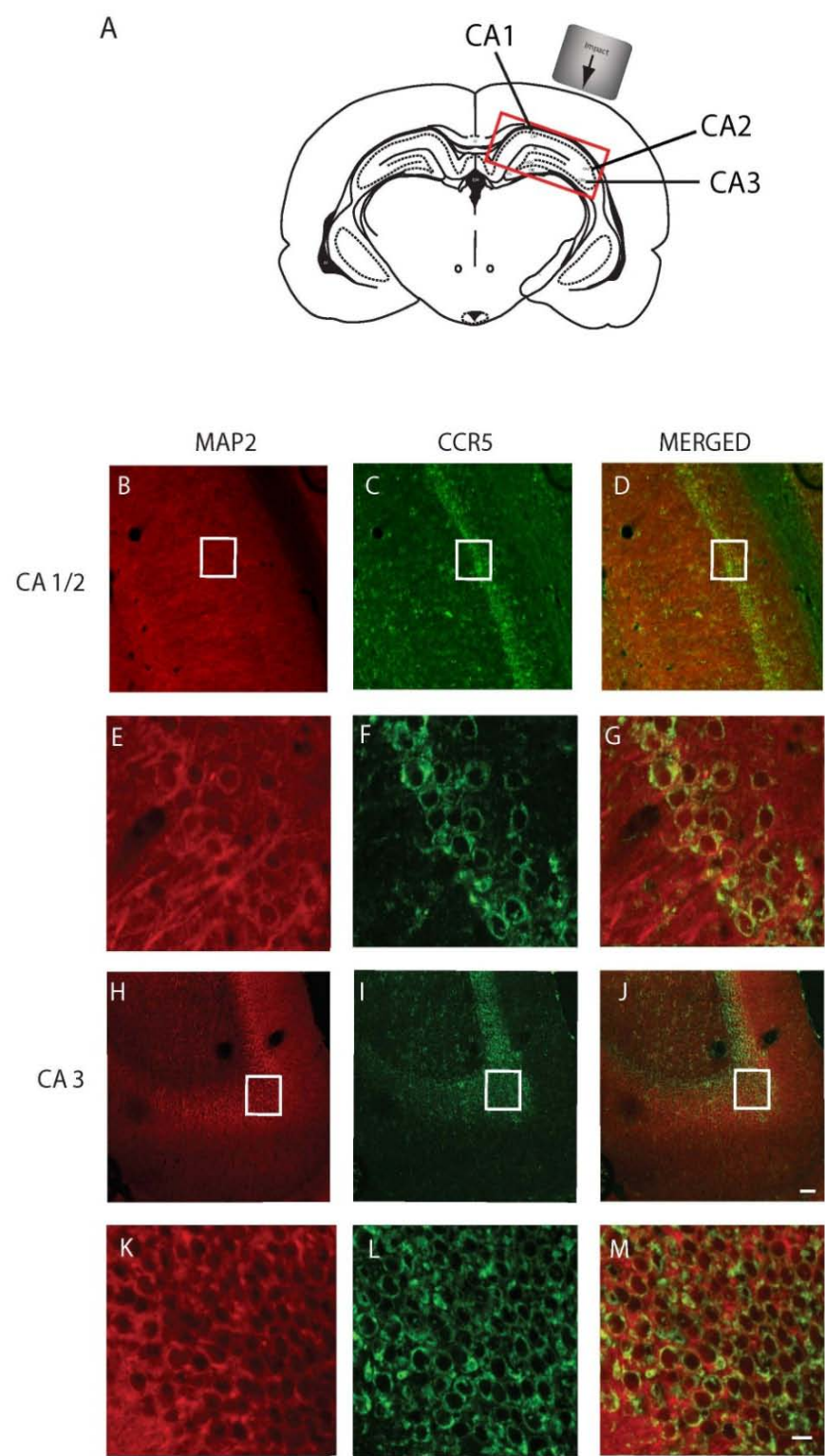


Fig 15. Immunohistochemical analysis of CCR5 protein expression in hippocampus 28 days post injury. Free-floating tissue sections were labeled with CCR5 (green) and MAP-2 (red). The region of interest for analysis is labeled with red rectangle (**A**). White boxes in low power images (**CA1/2: B-D**, **CA3: H-J**) indicate regions where high power images (**CA1/2: E-G**, **CA3: K-M**) were collected. Scale bar in J= 50 μ m and in M= 10 μ m.

Other areas of the brain were also observed for CCR5 expression (data not shown). CCR5 expression was observed to be in the cell bodies of neurons in the contralateral side of the brain; specifically in the cortex and hippocampus. CCR5 expression was also observed to be expressed in the corpus callosum. In the thalamus, CCR5 was observed to be expressed in the cell bodies of neurons as well.

6.4 NIH CCR5 Antibody Control Experiment

I used two different antibodies to label CCR5. For western analysis, I used a monoclonal CCR5 antibody (Santa Cruz, sc-17833) whereas for immunofluorescence, I was unable to detect any specific labeling using this antibody. I therefore used a function blocking CCR5 antibody that was obtained from the NIH Aids Research and Reference Reagent Program for immunolabeling. I performed a control experiment to assure that the NIH CCR5 antibody does recognize CCR5 labeling. HeLa cells were transfected with FLAG-CCR5 (Fig16 A-C) along with another chemokine receptor, HA-CXCR4 (Fig 16 D-F). To assure that the cells were transfected, I labeled the cells with antibodies of the appropriate tags. In addition, I also labeled the cells with the NIH CCR5 antibody to examine whether the antibody recognized the transfected CCR5 and not CXCR4.

Figure 16

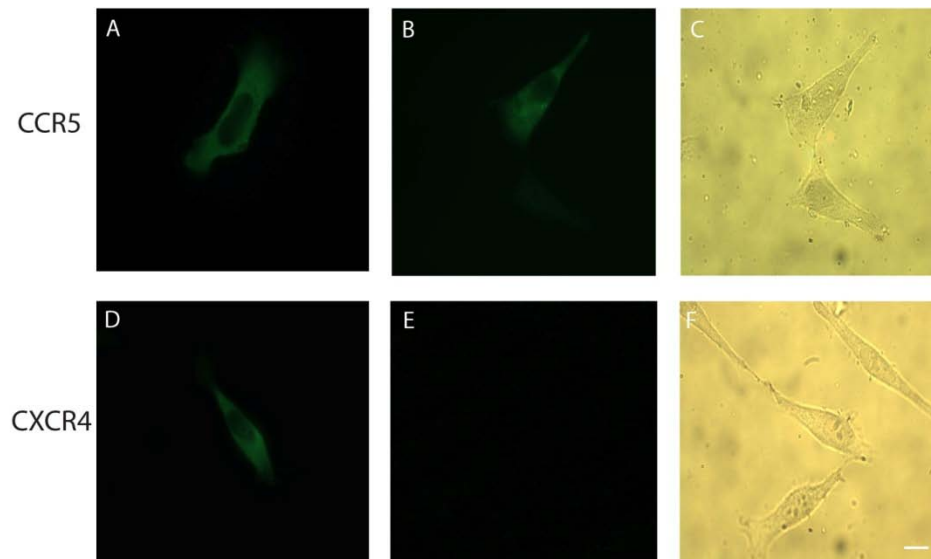


Fig 16. NIH CCR5 Antibody Control Experiment. HeLa cells were transfected with FLAG-CCR5 (A-C) or HA-CXCR4 (D-F). Cells were labeled with anti-FLAG antibody (A) or NIH CCR5 antibody (B). Panel C shows bright field image of cells present in panel B. Cells were labeled with HA antibody (D) or NIH CCR5 antibody (E). Panel F shows bright field image of cells that were present in panel C. Scale bar in F= 100 μ m.

DISCUSSION

In this study, I investigated the expression and cellular localization of CCR5 after traumatic brain injury. The expression of CCR5 mRNA and its ligands have previously been shown to be increased after various types of neural insult. For example, neonatal rats that received intra-hippocampal injections of N-methyl-D-aspartate (NMDA) displayed elevated levels of CCR5 mRNA expression in hippocampus as early as 16 hours after injection. CCR5 mRNA levels remained elevated up to 72 hours after injury while CCR5 protein expression was elevated 32 hours after injury (Galasso et al 1998). Cowell et al (2006) demonstrated that following hypoxia induced brain injury in neonatal rats, CCR5 mRNA levels were also increased with peak expression levels at 72 hours and 7 days after injury. These data suggest that the synthesis of CCR5 mRNA is increasing following injury. I found that CCR5 protein levels were increased in cortex and hippocampus of brain-injured rats in a time-dependent manner. Therefore, my data is in general agreement with previous reports.

Neuroinflammation takes place within hours to weeks after brain injury. A series of discrete events occur during this period that promote inflammation. For example, the breakdown of the blood brain barrier promotes inflammation by allowing the entry of circulating blood cells and neutrophils and lymphocytes that migrate towards the injured site and accumulate in the brain parenchyma. These immune cells become “activated” in response to injury. One aspect of this activation is that they begin to secrete inflammatory mediators such as pro- and anti-inflammatory cytokines as well as free radicals and complement factors. The release of these inflammatory mediators in the brain induces glial cells to secrete cytokines and chemokines. Secretion of these immune mediators within the brain parenchyma may produce additional neural damage but may also help to destroy the invading cells and help to repair

damaged tissue. My observation that CCR5 expression levels peaks rapidly after brain injury may be related to a role in CCR5 in an initial inflammatory event.

The time span of neural repair and recovery after brain injury ranges between days and weeks. In addition to chemokines and cytokines, activated astrocytes secrete trophic factors such as brain-derived growth factor (BDGF) and nerve growth factor (NGF) that can promote axonal regeneration and tissue repair. Glial cells can also secrete anti-inflammatory cytokines that can help promote neuronal repair by suppressing the production of pro-inflammatory cytokines. The observation that CCR5 protein expression increased at 14 days and 28 days after injury, presumably after the initial inflammatory event and may overlap with the time course for recovery phase suggests that CCR5 may have role in repair processes.

I found that CCR5 was localized on neurons in the brain before and after injury. Cowell et al (2006) observed that CCR5 was localized on activated microglia after hypoxia induced brain injury. The reason for the difference in localization between that study and mine is unclear although different antibodies were used in the two studies. We examined the antibody that Cowell et al used in their study on western blots of brain lysate and found that it recognized two bands in addition to the 46 kDa band that presumably corresponds to CCR5. We were not able to detect any specific labeling of brain tissue sections with that antibody. For immunolabeling experiments, we used an antibody obtained from the *NIH AIDS Research and Reference Reagent Program*. This antibody did not recognize any bands on western blots of brain lysate so we examined its specificity by transfecting HeLa cells with the cDNAs encoding CCR5 and the closely related cytokine receptor, and HIV co-receptor with CCR5, CXCR4. This antibody labeled the cell surface of cells transfected with the CCR5, but not CXCR4 suggesting that it can specifically recognize CCR5.

Chemokine binding to its receptor can activate multiple different downstream signaling pathways that result in a variety of cellular responses such as cell proliferation, cell adhesion,

chemotaxis, and polarization. The AKT/PKB signaling pathway has been shown to promote cellular survival and proliferation (81). Since CCR5 is a G-protein coupled receptor, the binding of chemokine to CCR5 leads to activation of a heterotrimeric G-proteins and dissociation of $G\alpha\beta\gamma$ subunits. Each G protein subunit can then activate different signaling pathways that eventually result in a physiological response. Specifically, the $\beta\gamma$ subunit activates PI3-kinase and subsequently the serine/threonine protein kinase AKT/PKB. AKT mediates cellular survival by blocking apoptosis through the phosphorylation of pro-apoptotic proteins such as BAD from inducing cellular death. In addition, AKT also regulates cellular survival through phosphorylation of specific transcription factors such as YAP (Yes-Associated Protein) thereby suppressing apoptosis (82). Because AKT is a major pathway that promotes cell survival, it may support CCR5's role in neuronal protection after brain injury.

One role for chemokines and their receptors is chemotaxis of migrating immune cells during inflammation. My observation that CCR5 is found in neurons might suggest a role for chemokine receptors in neuronal migration during brain development. Interestingly, CXCR4 knock-out mice had defects in cerebellum and hippocampus development due to migration defects of granule cells to form the layers required for proper brain development. In cerebellum development, CXCR4 knock-out mice resulted in disruption of the migration of granule cells to form the internal granule layer (83). Another study showed that a significant reduction in neuronal cell differentiation in CCR5 knock-out mice and the depletion of CCR5 using siRNA resulted in reduced differentiation of embryonic neuronal cells (84). These results suggest that CCR5 and its ligands are involved in the differentiation of embryonic neuronal cells during development. It is therefore possible that CCR5 may have a role in neuronal injury repair.

An in-vitro study by Gamo et al showed that the CCR5 ligand, RANTES (CCL5) was able to suppress inflammatory mediators through CCR5. Cultured microglial cells from mouse brain were stimulated with LPS (lipopolysaccharide) to activate microglia to produce pro-

inflammatory cytokines. When the cells were stimulated with RANTES, they observed a decrease in the production of mRNA for pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α . However, in CCR5 knock-out mice, RANTES does not suppress the pro-inflammatory cytokines suggesting that RANTES suppresses the production of pro-inflammatory cytokines through CCR5. Due to nerve injury, motor neuron death was also found accelerated in CCR5 knock-out mice (85). Therefore, CCR5 and its ligands may play a pro-survival role in neuronal protection after nerve injury. A role for CCR5 in development as well as a potential role in survival support my hypothesis that the increased expression of CCR5 in neurons after injury may be related to a role in neuronal protection.

CONCLUSION

In the context of this study, a role for CCR5 in development as well as recent findings in its potential role in survival may support the notion that CCR5 may be playing a role in neuronal protection after brain injury as aspects of injury repair recapitulate developmental processes. The observation that CCR5 is localized in neurons in both the cortex and hippocampus after brain injury also suggests a role for CCR5 in neuronal survival. It is possible that the chemokines of CCR5 may play a role in promoting neuronal protection through the suppression of other pro-inflammatory molecules. The activation of the AKT signal transduction pathway also promotes neuronal survival by inhibiting other proteins that mediate cellular death. Further studies in understanding the role of CCR5 in development as well as its signal transduction pathways in mediating cellular protection may contribute to further understanding of CCR5's potential mechanism in promoting neuronal survival.

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